Sensing of pathogens by Toll-like receptors in sarcoidosis

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Sensing of pathogens by Toll-like receptors in sarcoidosis

Het detecteren van pathogenen door Toll-like receptoren in sarcoïdose
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 22 februari 2011 des middags te 4.15 uur

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Drs. C.M. van Tilburg

Voor mijn geweldige ouders,

Martin† en Reina

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1

General introduction

1. SARCOIDOSIS

1.1 History

In January 1869, a 58-year-old coal-warf worker visited the British dermatologist J. Hutchinson complaining of purple symmetrical skin plaques on his legs and hands that had developed gradually over the preceding 2 years. The lesions were neither tender nor painful and Hutchinson described it as livid papillary psoriasis. In 1889, the French physician E. Besnier described a patient with violaceous swellings of the nose, ears and fingers, for which he proposed the term lupus pernio. He referred to J. Hutchinson's patient, but the distribution of the lesions was sufficiently dissimilar to justify his opinion that the two conditions were not identical. In 1899, after the Norwegian dermatologist C.Boeck published an extensive study of 24 cases of "benign miliary lupoids", it became clear that the curious skin lesions both found by Hutchinson and Besnier belonged to one multisystemic disease. The term for this disease, sarcoidosis, also known as Morbus Besnier-Boeck, was coined by Boeck to describe the clinical and histological features of this granulomatous disorder. It is derived from the Greek words "sark" and "oid" meaning "fleshy condition". Because in the opinion of Boeck this condition resembled sarcoma, he called the condition "multiple benign sarcoid of the skin" [1].

One century later, the descriptive definition of sarcoidosis, as reported by the American Thoracic Society/European Respiratory Society/World Association for Sarcoidosis and Other Granulomatous Disorders Statement on Sarcoidosis in 1999, reads as follows [2]. "Sarcoidosis is a multisystemic disorder of unknown cause. It commonly affects young and middle-aged adults and frequently presents with bilateral hilar lymphadenopathy, pulmonary infiltration, ocular and skin lesions. The liver, spleen, lymph nodes, salivary glands, heart, nervous system, muscles, bones and other organs may also be involved. The diagnosis is established when clinicoradiographic findings are supported by histologic evidence of non-caseating epitheloid cell granulomas. Granulomas of known causes must be excluded. Frequently observed immunologic features are depression of cutaneous delayed-type hypersensitivity and a heightened T-helper-1(Th1) immune response at sites of disease. Circulating immune complexes along with signs of B cell hyperactivity

may also be found. The course and prognosis may correlate with the mode of onset and extent of the disease. An acute onset with erythema nodosum or asymptomatic bilateral hilar adenopathy usually heralds a self-limiting course, whereas an insidious onset, especially with multiple extrapulmonary lesions, may be followed by relentless, progressively fibrosis of the lungs and other organs".

1.2 Epidemiology

Sarcoidosis is a disease of all races and ethnic groups with various incidences throughout the world [2]. It is most common in young adults between the ages of 20 and 40, with a number of studies suggesting a second peak after 50 years [3-5]. The highest incidence of sarcoidosis in Europe has been reported from Sweden (24 cases per 100.000) [6]. In The Netherlands, the incidence of sarcoidosis is estimated to be 20 cases per 100.000 [7]. In the United States, the incidence rate in black people is 36 cases per 100.000 compared to 11 cases per 100.000 in white people. Furthermore is has been demonstrated that sarcoidosis patients of African descent have more severe clinical manifestations and poorer outcomes compared to Caucasians [8]. Beside differences in incidence, differences in clinical phenotypes of sarcoidosis have been observed. Progression towards pulmonary fibrosis is more frequently seen in Caucasians and Negroid race than in Japanese patients [2]. Löfgren's syndrome is common in northern European countries but is rare in patients of African or Japanese origin [9-11]. On the other hand, uveitis, cardiac and skin involvement is more common in Japanese sarcoidosis patients [12]. The overall mortality is 1-5% and is caused by cardiac involvement in Japanese and respiratory failure in Caucasians[8].

1.3 Clinical presentation

There is an enormous variety in clinical presentation of sarcoidosis. Most patients present with symptoms such as fatigue, fever, dry cough, dyspnea, chest pain, malaise or weight loss [8]. Occasionally, sarcoidosis can be found in completely asymptomatic individuals. Pulmonary and mediastinal involvement is found in approximately 90% of the cases, but virtually every organ can be involved in this disease. Extrapulmonary organ involvement ranges from harmless skin manifestations

to life-threatening myocardial sarcoidosis or neurosarcoidosis [13,14]. Fortunately, in the majority of patients spontaneous remission occurs within 2-3 years. However, approximately 10-20% of the sarcoidosis cases develop pulmonary fibrosis with an unfavourable prognosis and increased mortality [15]. Unfortunately, the course of the disease is difficult to predict. One distinct clinical entity of sarcoidosis is known to have a favourable prognosis. It is characterized with fever, bilateral hilar lymphadenopathy, erythema nodosum and arthralgia and was first recognized by Sven Löfgren. Since then it has been known as Löfgren's syndrome [16].
Pulmonary involvement in sarcoidosis can be categorized using chest radiographs and is classified according to the Scadding criteria [2] (figure 1)

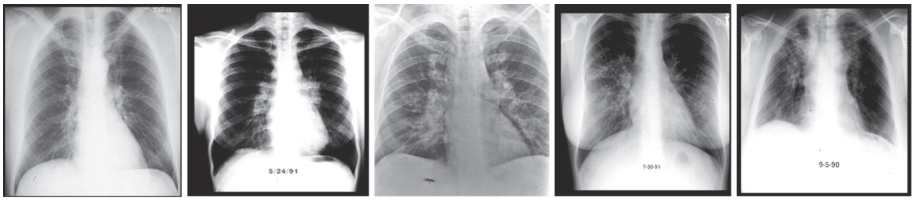

Figure 1. Staging of pulmonary sarcoidosis according to the Scadding criteria.
Stage 0 describes no visible intrathoracic findings. Stage I is bilateral hilar lymphadenopathy, which may be accompanied by paratracheal lymphadenopathy. Stage II is bilateral hilar lymphadenopathy accompanied by parenchymal infiltration. Stage III is parenchymal infiltration without hilar lymphadenopathy. Stage IV consists of advanced fibrosis with evidence of honeycombing, hilar retraction, bullae, cysts and emphysema.

Extrapulmonary involvement can be categorized using an Index Of Severity (IOS scale I-IV) as suggested by Grutters *et al.* [17]. IOS I is defined as organ manifestations that are harmless and without symptoms. IOS II means organ involvement that is in itself harmless but is associated with a substantial reduction in quality of life (e.g. lupus pernio). IOS III are extrapulmonary manifestations that cause severe loss of organ function and/or irreversible damage (e.g. posterior-segment involvement of the eye). Finally, IOS IV is associated with an increased risk of death (e.g. cardiac sarcoidosis).

1.4 Pathogenesis

The current models of sarcoidosis pathogenesis are based on data from patient studies, interpreted in the context of data from immunologic studies in mice [18-21]. The initial phase of sarcoidosis is thought to involve the exposure to still unknown exogenous or endogenous antigens which are taken up by antigen presenting cells in the lung. It is becoming clear that the first interaction between antigens and antigen presenting cells is important to orchestrate an adequate immune response. This first interaction, or innate immune recognition, will be discussed in more detail in the next paragraph. After processing into peptide fragments the antigen is loaded onto the binding groove of MHC (Major Histocompatibility Complex) class II molecules and presented to naïve CD-4+ T-lymphocytes (Th0 cells). This peptide-MHC complex is recognized by a T-cell receptor (TCR) adjacent to co-stimulatory molecules. This information of the MHC-peptide-TCR trimolecular complex and binding of co-stimulatory molecules give rise to signals essential for the activation of the T cell. Activated Th0 cells subsequently differentiate either into T-helper type I cells (Th1) or T-helper type 2 cells (Th2). This polarization is influenced by many different cytokines. Th1 cells support a cellular immune response which is necessary for intracellular pathogens such as viruses and intracellular bacteria or parasites. Th2 cells support a humoral immune response by activating B-cells to produce antibodies against extracellular pathogens. In sarcoidosis, activated Th0 cells are polarized to Th1 cells [20] (figure 2). Stimulation of Th1 cells and activation of macrophages leads to the production of IL-2, tumour necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ). Interestingly, there is evidence of oligoclonal expansion of T-cells expressing the T-cell receptor AV2S3 at sites of disease indicating that the inflammation in sarcoidosis indeed involves an antigen-driven response [22-24]. Ongoing presentation of antigens by macrophages to Th1 cells based on failure to eliminate the antigenic stimuli is believed to lead to formation of non-caseating granulomas, the pathological hallmark of sarcoidosis [25] (figure 3).

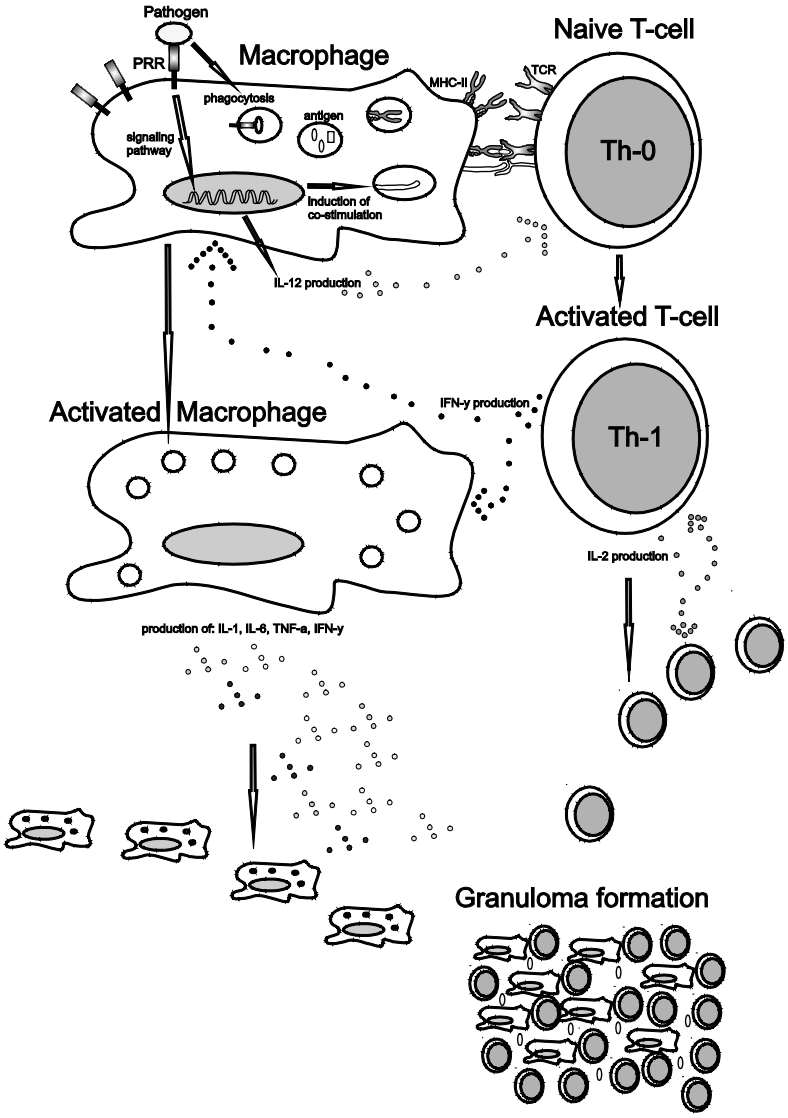

Figure 2. Induction of the primary immune response in sarcoidosis. An unknown antigen is recognized by pattern recognition receptors (PPRs) such as Toll-like receptors (TLRs), internalized and processed into peptides by antigen presenting cells (APC) such as macrophages. Peptides are loaded on major histocompatibility complex class II molecules (MHC-II) and presented to T-cell receptors (TCR) of CD4+ T-cells (Th0) in the presence of co-stimulatory molecules. Cytokines such as IL-12 polarise the Th0 cells to activated Th1 cells. These Th1 cells can activate macrophages by producing IFN-γ. Activated macrophages can produce IFN-γ as well as other cytokines such as TNF-α, important in granuloma formation.

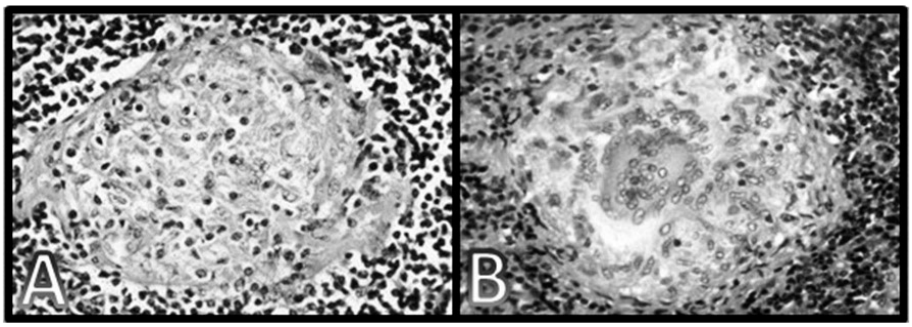

Figure 3. Non-caseating granuloma (A) and non-caseating granuloma with giant cell (B). Figures obtained from the Atlas of Granulomatous Diseases, http://granuloma.homestead.com

Granulomas are highly organized structures consisting of macrophages (which often fuse to form multinucleated giant cells), epitheloid cells and T cells. The function of granulomas is believed to sequester, and if possible, eliminate foreign antigens. Incomplete removal of the eliciting agents results in the maintenance of granulomas or even ongoing granuloma formation with persisting antigen presentation to innate and adaptive immune systems [20]. The latter may result in clinical deterioration of the disease. It is important to state that some of the cytokines involved in a Th1 response, e.g. TNF-α, are considered to play a role in the formation of granulomas [26]. This is also supported by the success of anti-TNF therapy in chronic sarcoidosis[27]. Granuloma formation is not only a feature of sarcoidosis, it can also be initiated by infectious agents such as *Mycobacterium tuberculosis* or fungi, or by noninfectious agents such as beryllium. Interestingly, the granuloma found in beryllium disease is identical to the one seen in sarcoidosis [28], implicating that sarcoidosis could reflect a collection of different granulomatous diseases, each with its own etiologic factor, rather than a single disease.

1.5 Etiological role of infectious agents

Analyses of sarcoidosis epidemiology indicate that transmissible agents have a role in its disease pathogenesis [8]. An increased risk for sarcoidosis was found in certain occupations such as agricultural employment, firemen and jobs which involve exposure to insecticides. Furthermore, there is an association between presentation

of Löfgren's disease and spring season[29] and it seems most likely that the agent is airborne [30]. The concept of a transmissible agent is strengthened by the fact that the Kveim-Siltzbach test, involving the subcutaneous injection of a homogenate of spleen or lymph nodes of sarcoidosis patients, was used to diagnose sarcoidosis in the previous century [31]. Oligoclonal expansion of T-cells, as mentioned earlier, was also found in the Kveim-induced granuloma, suggesting an antigen driven immune response [32]. Many potential organic/inorganic substances or micro organisms have been suggested to trigger sarcoidosis (table 1).

Category	Trigger
Infectious agents	*Propionibacterium acnes /P. granulosum* *Mycobacterium tuberculosis* Cell-wall deficient mycobacterial forms *Rickettsia helvetica* *Borrelia burgdorferi* Mycoplasma species Viruses (human herpes viruses, Epstein-Barr)
Inorganic substances	Aluminum Beryllium Zirconium Man-made mineral fibers Silica Silicone Clay Talc
Organic substances	Pine tree pollen Starch

Table 1. Potential triggers for sarcoidosis. Data from [2,33-39].

Currently, the most studied and strongest considered as causative agents are *Propionibacterium acnes* and *Mycobacterium tuberculosis*. *P. acnes* has been isolated from sarcoid lesions [40] and there seems a quantitative difference in *P.acnes* DNA between sarcoidosis lymph nodes and non-sarcoidosis lymph nodes [33]. Furthermore, it has been demonstrated that the number of *P.acnes* genomes found in Broncho Alveolar Lavage (BAL) cells of sarcoidosis patients is correlated with the angiotensin-converting enzyme (ACE) level in serum, a marker for disease

activity [41]. McCaskill et al. found that sensitization and challenge with *P. acnes* can produce granulomatous inflammation in the lung of mice, accompanied by an influx of CD4+ T-cells and Th1 cytokines[18]. Importantly, this response was not seen after sensitization and challenge using *Staphylococcus epidermidis*, another gram positive commensal of the human skin. In another study it was found that 35% of Japanese sarcoidosis patients (n=50) had an in-vitro response to a recombinant protein of *P. acnes,* found using a DNA expression library, while none of the healthy controls responded [42]. This recombinant protein, called RP35, was subsequently used in mice to demonstrate that experimentally induced hypersensitivity to RP35 or *P. acnes* can induce pulmonary granulomas [43]. Interestingly, there was an unexpected concordance in the rate of culture from normal mice lungs and the frequency of detection of pulmonary granulomas in sensitized mice (30%). This could suggest that granulomas can only be induced in mice having *P.acnes* in the normal indigenous flora of their lungs. In humans, it has also been suggested that *P.acnes* is a commensal of the lungs [44].

Due to clinical, radiological and pathological resemblance with tuberculosis numerous studies on *Mycobacterium tuberculosis* have been published addressing the potential role in sarcoidosis disease pathogenesis. Most studies report molecular evidence supporting an association of mycobacteria and sarcoidosis, nicely described in a recent meta-analysis [34]. Recent studies using interferon-gamma release assays (IGRA) demonstrated T-cell memory responses to certain mycobacterial antigens in sarcoidosis patients providing further evidence for a role of mycobacteria in disease pathogenesis [45,46].

Although most of these studies suggest a role for both bacteria in sarcoidosis, the inability to culture these micro organisms from pathological tissue continues to be one of the strongest arguments against a causal role for these agents. However, regarding Koch's postulates, it is important to emphasize that current culture and staining methods only identify less that 2% of current microbial communities present within human specimens [47,48].

2 INNATE IMMUNITY

The mammalian immune system consists of two different arms: innate and adaptive immunity. The innate immune system is an evolutionary conserved system that provides the first line of protection against pathogens and is mediated by macrophages, granulocytes, dendritic cells and natural killer (NK) cells [49-51]. This system works fast and efficiently. It is assumed that 90% of all infections are contained by the innate immune system. Adaptive immunity is highly specific, long lasting and has immunological memory, but is initially developed in the late phase of infection.

Micro organisms contain a wide range of invariable and highly conserved molecular patterns that differentiate these cells from eukaryotic cells of the host. These structures are called pathogen-associated molecular patterns (PAMPs) and are detected by cells of the innate immune system using as set of receptors called pattern recognition receptors (PRRs). This group of receptors contains scavenger receptors, Toll-like receptors, C-type lectin receptors, NOD-like (nucleotide-binding oligomerization domain-containing protein) receptors and soluble receptors such as mannose-binding lectin or sCD14 [20]. The recognition of PAMPs by PRRs is a key event in innate immunity and was first described in 1996 by Hoffmann an colleagues [52]. It was demonstrated that *Drosophila melanogaster* (fruit fly) carrying mutations in a protein important in embryonic dorso-ventral polarity formation were highly susceptible to fungal infection owing to defective induction of anti-fungal peptides. The researchers were so surprised that they shouted out in German "Das ist ja toll", which later became the name for this new (family of) receptors. Subsequently, human homologues of Drosophila Toll were discovered and named Toll-like receptors (TLRs). So far, 11 human and 13 murine TLRs have been identified [53,54]. TLRs are expressed differentially in a variety of cells and tissues, including innate immune cells and specific T and B cells. The receptors are either localized on the cellular membrane or within different cellular compartments. Ligands for these receptors consist of bacterial cell wall components, bacterial genome DNA, viral, fungal and parasitic products as well as endogenous molecules such as heat shock proteins, intercellular matrix products and mammalian genomic DNA. An overview of TLR ligands is given in table 2.

TLRs	Major cell types	Current recognized ligands	
		Exogenous	Endogenous
TLR1	Myeloid cells, T and B cells, NK cells, endothelial cells, epithelial cells, keratinocytes	Forms heterodimer with TLR2 for bacterial tri-acyl peptide, OSP of Borrelia spp and other ligands	
TLR2	Myeloid cells, T and B cells, NK cells, endothelial cells, epithelial cells, keratinocytes	Peptidoglycans, Mycoplasma lipopeptide, LAM from Mycobacteria BCG, LTA, GXM. LPS of spirochetes	HSP60, HSP79, HSP96, HMGB1
TLR3	Myeloid cells, T cells, NK cells, endothelial cells, epithelial cells, keratinocytes, neurons	Single-stranded viral RNA (ssRNA) and double strand RNA (dsRNA)	Self dsRNA
TLR4	Myeloid cells, NK cells, mast cells, T cells, endothelial cells, epithelial cells, keratinocytes	LPS, BCG, lipotechoid acid, respiratory syncytial virus, fibronectin, heparin sulfate, paclitaxel	HSP22, HSP60, HSP70, HSP96, HMGB1, β-defensin 2
TLR5	Myeloid cells, T cells, NK cells, endothelial cells, epithelial cells, keratinocytes	Flagellin	
TLR6	Myeloid cells, T and B cells, NK cells, endothelial cells, epithelial cells, keratinocytes	Forms heterodimers with TLR2 for Mycoplasma di-acyl-lipopeptides, peptidoglycan from Gram-positive bacteria, Zymosan	
TLR7	Myeloid cells, NK cells, endothelial cells, T and B cells	Single-strand RNA, imidazoguinolines	Self ssRNA
TLR8	Myeloid cells, NK cells, endothelial cells	Single-strand RNA	Self ssRNA
TLR9	Myeloid cells, T and B cells, NK cells, endothelial cells, epithelial cells, keratinocytes	Unmethylated CpG motifs found in microbial DNA	Self DNA
TLR10	Myeloid cells, T and B cells, endothelial cells, epithelial cells	Unknown, suggested to form heterodimer with TLR2	
TLR11	Epithelial cells, myeloid cells	Profilin	unknown

Table 2. Human Toll-like receptors (TLRs) and their principal ligands. Data obtained from a review by Chang *et al.* [62] and [63]. CpG cytidine-phosphate-guanine, GXM glucuronoxylomannan, HSP heat shock protein, LAM lipoarabinomannan, LPS lipopolysaccharide, LTA lipoteichoic acid. BCG Bacillus Calmette-Guérin, NK natural killer, OSP outer surface protein, HMGB1 high mobility group box 1.

Upon binding with their ligands, TLRs activate major signaling pathways via NF-κβ or transcription factor IRF (IFN regulatory factor) to (1) induce pro inflammatory cytokines and chemokines such as IL-12, TNF-α, IL-1, IL-6 and IL-8, (2) increase phagocytosis of micro organisms, induce killing through intracellular production of reactive oxygen and nitrogen intermediates, (3) stimulate T and B cells to mediate adaptive immune responses and (4), play a critical role in tissue injury and tissue repair and regeneration by a TLR dictated complex regulatory process of cell proliferation, survival and apoptosis.
It has been well established that the innate immune system is of prime importance for the onset and regulation of the adaptive immune response. For granulomatous disorders such as chronic granulomatous disease, juvenile sarcoidosis (in literature also described as early-onset sarcoidosis), tuberculosis, leprosy and Crohn's disease, defects in innate immune responses have been suggested to play a role in disease pathogenesis [55-61]. This indicates that innate immunity could also be involved in sarcoidosis disease pathogenesis.

3 AIM OF THIS THESIS

The underlying hypothesis of this thesis is that genetic and functional defects in Toll-like receptors are involved in the innate immune recognition of intracellular bacteria in sarcoidosis, and play a role in disease susceptibility and clinical outcome. Furthermore, we hypothesize that mycobacteria and propionibacteria are possible antigenic triggers in sarcoidosis patients in The Netherlands.

4 SCOPE AND OUTLINE OF THIS THESIS

Chapter 2 describes the genetic variation of an important TLR-4 single nucleotide polymorphism (SNP) in sarcoidosis patients and matched controls.

In **Chapter 3** genetic variation and functional consequences of 3 SNPs and one tandem repeat polymorphism in TLR-2 are analyzed in sarcoidosis patients and

healthy controls. Each genotype was evaluated for association with radiographic evolution of disease, as wells as the distinct clinical phenotype of Löfgren's syndrome.

Chapter 4 presents the genotyping and haplotype formation of 4 SNPs in the promoter region of the gene encoding for CD14. Sarcoidosis patients with different pulmonary disease phenotypes, according to radiographic evolution, and patients with Löfgren's sydrome are compared in terms of SNP and haplotype frequency distributions.

Chapter 5 evaluates possible genetic or functional differences in TLR-9 between sarcoidosis patients and healthy controls. Allelic distribution of 4 SNPs are shown as well as sequence data on part of the promoter in combination with both exons in order to identify new mutations. Data are completed with in vitro experiments on TLR-9 function.

In **chapter 6,** a total of 8 SNPs present in the TLR10-TLR1-TLR6 gene cluster are investigated in sarcoidosis patients. Separate analysis of patients with self-remitting or chronic disease was performed in terms of SNP and haplotype frequency distributions.

Chapter 7 describes the result of interferon-gamma release assays using antigens from *M. tuberculosis*, *P. acnes* and *P. granulosum* in a small cohort of sarcoidosis patients. A perspective is given on the importance of identifying new disease phenotypes within sarcoidosis.

Chapter 8 summarizes and provides concluding remarks.

Reference List

1. C.Boeck. Mutiple benign sarcoid of the skin. *J. Cutan. Genitourin. Dis* **17**, 543-550 (1899).
2. Statement on sarcoidosis. Joint Statement of the American Thoracic Society (ATS), the European Respiratory Society (ERS) and the World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG) adopted by the ATS Board of Directors and by the ERS Executive Committee, February 1999. *Am. J. Respir. Crit Care Med.* **160**, 736-755 (1999).
3. Henke, C. E. *et al.* The epidemiology of sarcoidosis in Rochester, Minnesota: a population-based study of incidence and survival. *Am. J. Epidemiol.* **123**, 840-845 (1986).
4. Hillerdal, G., Nou, E., Osterman, K. & Schmekel, B. Sarcoidosis: epidemiology and prognosis. A 15-year European study. *Am. Rev. Respir. Dis* **130**, 29-32 (1984).
5. Pietinalho, A., Ohmichi, M., Lofroos, A. B., Hiraga, Y. & Selroos, O. The prognosis of pulmonary sarcoidosis in Finland and Hokkaido, Japan. A comparative five-year study of biopsy-proven cases. *Sarcoidosis. Vasc. Diffuse. Lung Dis* **17**, 158-166 (2000).
6. Milman, N. & Selroos, O. Pulmonary sarcoidosis in the Nordic countries 1950-1982. Epidemiology and clinical picture. *Sarcoidosis.* **7**, 50-57 (1990).
7. Lieke S.J.Kamphuis. Sarcoïdose: verandering in therapeutische inzichten. *Ned. Tijdschr. Geneeskd.* **154**, A1685 (2010).
8. Baughman, R. P. *et al.* Clinical characteristics of patients in a case control study of sarcoidosis. *Am. J. Respir. Crit Care Med.* **164**, 1885-1889 (2001).
9. Grunewald, J. & Eklund, A. Human leukocyte antigen genes may outweigh racial background when generating a specific immune response in sarcoidosis. *Eur. Respir. J.* **17**, 1046-1048 (2001).
10. Higashimoto, I., Arata, S. I. & Niena, K. [A case of acute sarcoidosis with polyarthralgia and erythema nodosum; Lofgren's syndrome]. *Nihon Kokyuki. Gakkai Zasshi* **40**, 679-682 (2002).
11. Koseki, Y. *et al.* [A patient with acute sarcoidosis associated with fever, polyarthritis, and erythema nodosum: a typical of Lofgren's syndrome]. *Ryumachi* **38**, 23-28 (1998).
12. Iwai, K. *et al.* Racial difference in cardiac sarcoidosis incidence observed at autopsy. *Sarcoidosis.* **11**, 26-31 (1994).
13. Perry, A. & Vuitch, F. Causes of death in patients with sarcoidosis. A morphologic study of 38 autopsies with clinicopathologic correlations. *Arch. Pathol. Lab Med* **119**, 167-172 (1995).
14. Sharma, O. P. Neurosarcoidosis: a personal perspective based on the study of 37 patients. *Chest* **112**, 220-228 (1997).
15. Crystal, R. G., Bitterman, P. B., Rennard, S. I., Hance, A. J. & Keogh, B. A. Interstitial lung diseases of unknown cause. Disorders characterized by chronic inflammation of the lower respiratory tract. *N. Engl. J. Med.* **310**, 235-244 (1984).

16. Löfgren S. Erythema nodosum: studies on etiology and pathogenesis in 185 adult cases. *Acta Med Scand 1946* **124**, 1-197

17. Grutters JC.Drent M. & van den Bosch JMM. Sarcoidosis. *Eur. Respir. Monograph.* **46**, 126-154 (2009).

18. McCaskill, J. G. *et al.* Pulmonary immune responses to Propionibacterium acnes in C57BL/6 and BALB/c mice. *Am. J. Respir. Cell Mol. Biol.* **35**, 347-356 (2006).

19. Iio, K. *et al.* Experimental pulmonary granuloma mimicking sarcoidosis induced by Propionibacterium acnes in mice. *Acta Med. Okayama* **64**, 75-83 (2010).

20. Zissel, G., Prasse, A. & Muller-Quernheim, J. Immunologic response of sarcoidosis. *Semin. Respir. Crit Care Med* **31**, 390-403 (2010).

21. Swaisgood, C. M. *et al.* Development of a Sarcoidosis Murine Lung Granuloma Model Using Mycobacterium sodA. *Am. J. Respir. Cell Mol. Biol.* (2010).

22. Grunewald, J. *et al.* Restricted V alpha 2.3 gene usage by CD4+ T lymphocytes in bronchoalveolar lavage fluid from sarcoidosis patients correlates with HLA-DR3. *Eur. J. Immunol.* **22**, 129-135 (1992).

23. Grunewald, J., Berlin, M., Olerup, O. & Eklund, A. Lung T-helper cells expressing T-cell receptor AV2S3 associate with clinical features of pulmonary sarcoidosis. *Am. J. Respir. Crit Care Med* **161**, 814-818 (2000).

24. Moller, D. R. Involvement of T cells and alterations in T cell receptors in sarcoidosis. *Semin. Respir. Infect.* **13**, 174-183 (1998).

25. Thomas, P. D. & Hunninghake, G. W. Current concepts of the pathogenesis of sarcoidosis. *Am. Rev. Respir. Dis* **135**, 747-760 (1987).

26. Agostini, C., Adami, F. & Semenzato, G. New pathogenetic insights into the sarcoid granuloma. *Curr. Opin. Rheumatol.* **12**, 71-76 (2000).

27. Baughman, R. P. *et al.* Infliximab therapy in patients with chronic sarcoidosis and pulmonary involvement. *Am. J. Respir. Crit Care Med* **174**, 795-802 (2006).

28. Muller-Quernheim, J., Gaede, K. I., Fireman, E. & Zissel, G. Diagnoses of chronic beryllium disease within cohorts of sarcoidosis patients. *Eur. Respir. J.* **27**, 1190-1195 (2006).

29. Wilsher, M. L. Seasonal clustering of sarcoidosis presenting with erythema nodosum. *Eur. Respir. J.* **12**, 1197-1199 (1998).

30. Bardinas, F., Morera, J., Fite, E. & Plasencia, A. Seasonal clustering of sarcoidosis. *Lancet* **2**, 455-456 (1989).

31. Munro, C. S. & Mitchell, D. N. The K veim response: still useful, still a puzzle. *Thorax* **42**, 321-331 (1987).

32. Klein, J. T. *et al.* Selection of oligoclonal V beta-specific T cells in the intradermal response to Kveim-Siltzbach reagent in individuals with sarcoidosis. *J. Immunol.* **154**, 1450-1460 (1995).

33. Eishi, Y. *et al.* Quantitative analysis of mycobacterial and propionibacterial DNA in lymph nodes of Japanese and European patients with sarcoidosis. *J. Clin. Microbiol.* **40**, 198-204 (2002).

34. Gupta, D., Agarwal, R., Aggarwal, A. N. & Jindal, S. K. Molecular evidence for the role of mycobacteria in sarcoidosis: a meta-analysis. *Eur. Respir. J.* **30**, 508-516 (2007).

35. Baughman, R. P., Lower, E. E. & du Bois, R. M. Sarcoidosis. *Lancet* **361**, 1111-1118 (2003).

36. Nilsson, K. *et al.* Presence of Rickettsia helvetica in granulomatous tissue from patients with sarcoidosis. *J. Infect. Dis* **185**, 1128-1138 (2002).

37. Rafnsson, V., Ingimarsson, O., Hjalmarsson, I. & Gunnarsdottir, H. Association between exposure to crystalline silica and risk of sarcoidosis. *Occup. Environ. Med* **55**, 657-660 (1998).

38. Drent, M. *et al.* Association of man-made mineral fibre exposure and sarcoidlike granulomas. *Respir. Med* **94**, 815-820 (2000).

39. Sola, R., Boj, M., Hernandez-Flix, S. & Camprubi, M. Silica in oral drugs as a possible sarcoidosis-inducing antigen. *Lancet* **373**, 1943-1944 (2009).

40. Abe, C., Iwai, K., Mikami, R. & Hosoda, Y. Frequent isolation of Propionibacterium acnes from sarcoidosis lymph nodes. *Zentralbl. Bakteriol. Mikrobiol. Hyg. A* **256**, 541-547 (1984).

41. Ichikawa, H. *et al.* Quantitative analysis of propionibacterial DNA in bronchoalveolar lavage cells from patients with sarcoidosis. *Sarcoidosis. Vasc. Diffuse. Lung Dis* **25**, 15-20 (2008).

42. Ebe, Y. *et al.* Proliferative response of peripheral blood mononuclear cells and levels of antibody to recombinant protein from Propionibacterium acnes DNA expression library in Japanese patients with sarcoidosis. *Sarcoidosis. Vasc. Diffuse. Lung Dis* **17**, 256-265 (2000).

43. Minami, J. *et al.* Pulmonary granulomas caused experimentally in mice by a recombinant trigger-factor protein of Propionibacterium acnes. *J. Med Dent. Sci.* **50**, 265-274 (2003).

44. Ishige, I. *et al.* Propionibacterium acnes is the most common bacterium commensal in peripheral lung tissue and mediastinal lymph nodes from subjects without sarcoidosis. *Sarcoidosis. Vasc. Diffuse. Lung Dis* **22**, 33-42 (2005).

45. Chen, E. S. *et al.* T cell responses to mycobacterial catalase-peroxidase profile a pathogenic antigen in systemic sarcoidosis. *J. Immunol.* **181**, 8784-8796 (2008).

46. Drake, W. P. *et al.* Cellular recognition of Mycobacterium tuberculosis ESAT-6 and KatG peptides in systemic sarcoidosis. *Infect. Immun.* **75**, 527-530 (2007).

47. DiGiulio, D. B. *et al.* Microbial prevalence, diversity and abundance in amniotic fluid during preterm labor: a molecular and culture-based investigation. *PLoS. One.* **3**, e3056 (2008).

48. Bik, E. M. *et al.* Molecular analysis of the bacterial microbiota in the human stomach. *Proc. Natl. Acad. Sci. U. S. A* **103**, 732-737 (2006).

49. Medzhitov, R. Recognition of microorganisms and activation of the immune response. *Nature* **449**, 819-826 (2007).

50. Akira, S., Uematsu, S. & Takeuchi, O. Pathogen recognition and innate immunity. *Cell* **124**, 783-801 (2006).

51. Hoffmann, J. A. The immune response of Drosophila. *Nature* **426**, 33-38 (2003).

52. Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J. M. & Hoffmann, J. A. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell* **86**, 973-983 (1996).

53. Ishii, K. J., Koyama, S., Nakagawa, A., Coban, C. & Akira, S. Host innate immune receptors and beyond: making sense of microbial infections. *Cell Host. Microbe* **3**, 352-363 (2008).

54. McGettrick, A. F. & O'Neill, L. A. Toll-like receptors: key activators of leucocytes and regulator of haematopoiesis. *Br. J. Haematol.* **139**, 185-193 (2007).

55. Marks, D. J. *et al.* Defective acute inflammation in Crohn's disease: a clinical investigation. *Lancet. 2006 Feb* **25;367**, 668-678.

56. Bafica, A. *et al.* TLR9 regulates Th1 responses and cooperates with TLR2 in mediating optimal resistance to Mycobacterium tuberculosis. *J Exp Med. 2005 Dec* **19;202**, 1715-1724.

57. Ogus, A. C. *et al.* The Arg753GLn polymorphism of the human toll-like receptor 2 gene in tuberculosis disease. *Eur Respir J. 2004 Feb* **23**, 219-223.

58. Yim, J. J. *et al.* The association between microsatellite polymorphisms in intron II of the human Toll-like receptor 2 gene and tuberculosis among Koreans. *Genes Immun. 2006 Mar* **7**, 150-155.

59. Barreiro, L. B. *et al.* Promoter variation in the DC-SIGN-encoding gene CD209 is associated with tuberculosis. *PLoS. Med* **3**, e20 (2006).

60. Sapkota, B. R. *et al.* Association of TNF, MBL, and VDR polymorphisms with leprosy phenotypes. *Hum. Immunol.* **71**, 992-998 (2010).

61. Berrington, W. R. *et al.* Common polymorphisms in the NOD2 gene region are associated with leprosy and its reactive states. *J. Infect. Dis* **201**, 1422-1435 (2010).

62. Chang, Z. L. Important aspects of Toll-like receptors, ligands and their signaling pathways. *Inflamm. Res.* **59**, 791-808 (2010).

63. Cai, Z. *et al.* Transcriptional regulation of Tlr11 gene expression in epithelial cells. *J. Biol. Chem.* **284**, 33088-33096 (2009).

2

Toll-like receptor (TLR) 4 polymorphism Asp299Gly is not associated with disease course in Dutch sarcoidosis patients

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Summary

The aetiology of sarcoidosis, a systemic disorder characterized by the formation of noncaseating granulomas in variable organs, remains enigmatic. Clarification is hampered by heterogeneity in disease phenotypes and course, partly due to the influence of a variety of genetic and environmental factors. Multiple studies have pointed towards bacteria as possible causative agents. Toll-like receptors (TLR) are innate immunity receptors important in the immune response against pathogens. TLR-4, together with CD14 and MD-2, is an essential receptor for the recognition of LPS, unique to the cell wall of gram-negative bacteria.

Recently, an association between TLR-4 polymorphism Asp299Gly, leading to a change in the extracellular domain of the receptor and possible hyporesponsiveness to LPS, and a chronic course of sarcoidosis was found in German patients. In the present study this polymorphism was genotyped in 156 Dutch sarcoidosis patients and 200 healthy Dutch controls using dual labelled fluorescent oligonucleotides. No differences were found in allelic distributions between patients and controls ($p=0.79$) or within the different clinical entities of the sarcoidosis group ($p=0.44$). Importantly, there were no differences between the Dutch and German sarcoidosis patients ($p=0.62$). However, the allelic distribution of the Asp299Gly polymorphism differed significantly between both control groups ($p=0.04$). This study highlights the importance of testing a reported gene association in a distinct population when performing genetic association studies.

Introduction

Sarcoidosis is a systemic disorder characterized by the formation of noncaseating granulomas in variable organs, usually the lungs. In half of the cases, sarcoidosis follows a benign course with spontaneous resolution within 12-36 months[1]. However, the disease can be severe due to localisation of granulomas in particularly vulnerable organs such as the central nervous system or heart, or as a consequence of the abundance of granulomas in the lungs leading to respiratory insufficiency. Mortality attributable to sarcoidosis is estimated between 0.5 and 5%[2] which is clinically relevant for a disease affecting relatively young people.

The aetiology of sarcoidosis remains unknown, which has been attributed to its heterogeneity in clinical phenotypes that might reflect a collection of different granulomatous diseases, each with its own etiologic factor, rather than a single disease. Sarcoidosis is characterized by a strong cell-mediated immune reaction, which is essential for combating viruses or intracellular bacteria. The possible role of bacteria in sarcoidosis has therefore been extensively studied. Especially Gram-positive and intracellular bacteria, such as mycobacteria and propionibacteria, have been suggested to play a role in the aetiology of sarcoidosis[3,4]. There is no convincing evidence, however, on a possible role for Gram-negative bacteria in sarcoidosis[5].

Toll-like receptors (TLRs) are innate immunity receptors responsible for the molecular recognition of pathogens. TLRs can initiate inflammatory and anti-microbial innate immune response, thereby dictating the ensuing adaptive immune response. TLR-2 and TLR-4 are the most studied among the TLR family. TLR-2 recognizes a variety of microbial components, such as lipoproteins, peptidoglycan and lipoteichoic acid from Gram-positive bacteria and lipoarabinomannan from mycobacteria, zymosan from fungi and some glycolipids[6]. TLR-4 is an essential receptor for the recognition of LPS, unique to the cell wall of gram-negative bacteria[7].

Thus when considering a causative role of bacteria in the pathogenesis of sarcoidosis, TLRs recognising microbial components of Gram-positive bacteria, and not TLR-4, might be good candidates for genetic association studies in sarcoidosis. Surprisingly, Pabst et al recently published an article in this journal where they

demonstrate an association between the TLR-4 polymorphisms Asp299Gly and Thr399Ile and the chronic course of sarcoidosis[8]. These results could indicate that Gram-negative bacteria might nevertheless be candidates for a bacterial agent in sarcoidosis.
In this study, we tried to confirm the association between the TLR-4 polymorphisms and sarcoidosis in Dutch patients. Since Asp299Gly and Thr399Ile alleles cosegregate, and a functional alteration of the receptor was only proven for the Asp299Gly mutation[9], we examined the functional polymorphism in our cohort.

Materials and Methods

Subjects

156 unrelated and randomly selected Dutch white patients with sarcoidosis (88 men and 67 women) were included in the study. In 112 patients, the diagnosis of sarcoidosis was established when clinical findings were supported by histological evidence, and after the exclusion of other known causes of granulomatosis. 44 patients presented with the classical Löfgren's syndrome of fever, erythema nodosum, bilateral hilar lymphadenopathy and arthritis. The diagnosis in these patients was mostly made without biopsy proof [10]. Verbal and written consent was obtained from all subjects, and authorization was given by the Ethics Committee of the St. Antonius Hospital, Nieuwegein. The control subjects comprised 200 healthy Dutch Caucasian employees of the St Antonius Hospital in the Netherlands (118 men and 82 women). By completing a questionnaire, relevant background information was provided by these volunteers and included medication, ethnicity and hereditary diseases.

Evaluation of pulmonary disease severity

Pulmonary disease severity at presentation was evaluated by chest radiography. Further chest radiographs for each patient were examined and compared to determine disease outcome. Chest radiographs at presentation, 2 years and 4 years were collected for each patient and assessed blind by a pulmonary physician

for disease severity using standard radiographic staging for sarcoidosis. In brief, this comprises five stages: stage 0, normal, stage I, bilateral hilar lymphadenopathy (BHL), stage II, BHL and parenchymal infiltration, stage III, parenchymal infiltration without BHL, stage IV, irreversible fibrosis with loss of lung volume. Radiographic evolution over a 4-year period was available for all 156 patients. In the statistical analysis acute sarcoidosis was defined as Löfgren's syndrome or a normalized chest X-ray 2 years after presentation. Chronic sarcoidosis was defined as a disease course over at least 2 years.

Analysis of the TLR-4 Asp299Gly polymorphism

Genomic DNA from all subjects and controls, extracted from peripheral blood cells, was genotyped for the Asp299Gly polymorphism using single tube PCR and exonuclease degradation of dual labeled fluorescent oligonucleotides as described elsewhere[11]

Statistical analysis

Allele and genotype frequencies were calculated and tested for Hardy-Weinberg equilibrium in controls. Association studies were analysed by chi-square testing using 2x3 and 2x2 contingency tables for genotype and allele frequencies, respectively. A p value <0.05 was considered significant.

Results

The genotype of the investigated Asp299Gly polymorphism in Dutch sarcoidosis and control populations are presented, together with the data of the German sarcoidosis study by Pabst et al[12], in table 1.

		No.	AA	AG	GG	G allele frequency
Sarcoidosis (all patients)	Dutch	156	135	21	0	6.7
	German	141	119	22	0	7.8
Acute sarcoidosis	Dutch	62	52	10	0	8.0
	German	33	31	2	0	3.0
Chronic sarcoidosis	Dutch	94	83	11	0	5.9
	German	108	88	20	0	9.3
Controls	Dutch	200	176	23	1	6.3
	German	141	133	8	0	2.8

Table 1. Prevalence of different TLR-4 Asp299Gly genotypes in Dutch sarcoidosis patients and controls. The results of the German population are extracted from the article published by Pabst et al[13]. No. = number. G Allele frequency is shown as a percentage.

Genotype data from all populations were in Hardy-Weinberg equilibrium. Among 200 healthy Dutch controls, 23 were heterozygous for Asp299Gly and 1 was homozygous for the minor allele (minor allele frequency 6,3%; 25/400, table 1). Among the sarcoidosis patients, 21 were heterozygous and no homozygous mutation was found (minor allele frequency 6,7%; 21/312, table 1). No differences were found in allelic distributions between patients and controls (p= 0.79) or when comparing the different clinical entities within the sarcoidosis group (p= 0.44). Also, when comparing the Dutch sarcoidosis patients with the German sarcoidosis patients, no difference in allelic distributions was found (p= 0.62). However, Asp299Gly polymorphisms occurred more frequently in Dutch controls compared to German controls (p= 0.04).

Discussion

TLR-4 is an innate immunity pattern recognition receptor expressed on a variety of human cells, such as monocytes, mast cells, neutrophils, dendritic cells, T cells and endothelial cells. TLR-4 is an essential receptor for the recognition of LPS, unique to the cell wall of gram-negative bacteria. However, the main LPS binding receptor is CD14, also a pattern recognition receptor[14]. TLR-4 acts as a co-receptor for CD14, together with MD-2, and is responsible for activating intracellular signaling

pathways resulting in the production of proinflammatory cytokines and upregulation of costimulatory molecules, thereby leading to priming of an adaptive immune response[15].

In the present study, we tried to confirm a recent report of an association between the TLR-4 polymorphism Asp299Gly and severity of sarcoidosis in a clinically well-defined population from the Netherlands. At the genomic location of this polymorphism, an A to G substitution leads to the replacement of aspartic acid with glycine at amino acid 299, located in the extracellular domain of TLR-4. This substitution has been associated with LPS hyporesponsiveness in humans[16], however, some strong conflicting studies have been published[17,18].

We did not find an association between the allelic distribution of the Asp299Gly polymorphism in sarcoidosis and controls, nor between different clinical subsets of disease severity. Comparing the data of multiple studies regarding the role of bacteria in initiating the immune response in saroidosis, it is to be expected that a pattern recognition receptor involved in the immune response against Gram-negative bacteria will not likely play a role. To our opinion these results are therefore in line with current concepts of the role of bacteria in the etiopathogenesis of sarcoidosis.

But, TLR-4 has also been shown to be involved in the recognition of endogenous ligands such as heat shock proteins (HSP60 and HSP70)[19] and cross-reactivity with mycobacterial heat shock proteins is suggested as disease mechanism in sarcoidosis[20]. Therefore, a possible role for TLR-4 in this disease can still not fully be excluded.

Importantly, this study again demonstrates the importance in studying multiple populations in the search for genetic variants in sarcoidosis. Pabst et al[21] found a significant association between patients with a chronic course of sarcoidosis and the Asp299Gly polymorphism, while this association could not be replicated in another white European sarcoidosis cohort. The allele frequency of this polymorphism was almost the same in Dutch and German sarcoidosis patients. However, a remarkable and significant difference was found when comparing the allele frequency in both healthy control populations. The minor allele of the Asp299Gly polymorphism occurred more frequently in Dutch controls compared to German controls. This discrepancy between these control groups is striking.

In contrast with the restricted fragment length polymorphism (RFLP) analysis used by Pabst and colleagues[22], in the present study the Aps299Gly polymorphism was determined with dual labelled allele-specific oligonucleotides. Van Rijn et al[23] compared both techniques and demonstrated that they reveal identical Asp299Gly genotyping results, indicating that this cannot explain the observed differences between the Dutch and German controls. Furthermore, it is well known that allele frequencies of single nucleotide polymorphisms, such as Asp299Gly, vary between different ethnic populations. The TLR-4 Asp299Gly polymorphism has different minor allele frequencies in for example Chinese (0%) [24], American (6.4%)[25] and West-African populations (9.8%) [26]. An explanation for the discrepancy therefore could be, although not very likely, that there is a difference in Asp299Gly minor allele carriership between The Netherlands and Germany. We searched for recent publications with control groups over 150 subjects in order to compare Dutch and German Asp299Gly minor allele frequencies (table 2). No differences in allele frequencies were seen between healthy controls from The Netherlands and Germany (5.9% vs. 5.4% respectively). Furthermore, our results on the Asp299Gly minor allele frequency are comparable with other healthy Dutch controls (table 2).

Study	Country	Control population (no.)	Allele frequency
Present study	Netherlands	200	6.3
Netea et al[27]	Netherlands	200	5.5
Hawn et al [28]	Netherlands	495	6.5
Ouburg et al [29]	Netherlands	170	5.3
Van der Paardt et al [30]	Netherlands	169	4.7
Pabst et al [31]	Germany	141	2.8
Kroner et al [32]	Germany	350	4.4
Reismann et al[33]	Germany	343	5.1
Brand et al [34]	Germany	199	3.8
Rohde et al [35]	Germany	444	7.8

Table 2. Minor allele frequency of the Asp299Gly polymorphism in different Dutch and German control groups from several published studies. Allele frequency is shown as a percentage. no = number.

The Asp299Gly minor allele frequency found by Pabst et al seems, as they mention in their article, slightly lower than other healthy German controls (table 2). Therefore, the calculation of allele frequency using relatively small numbers seems susceptible for under- or overestimation, stressing the importance of being reserved when drawing conclusions.

In conclusion, the TLR-4 Asp299Gly is not associated with sarcoidosis or disease course in Dutch subjects. Therefore, it is unlikely that TLR-4 genetic variants play a major role in sarcoidosis pathogenesis. However, before excluding a role for this receptor, functional data, such as influences of this or other TLR-4 polymorphisms on TLR-4 cell surface expression and interaction with pathogens, are necessary. Also, the influence of CD14 or MD-2 should be studied. Importantly, this study highlights one of the most critical aspects of genetic association studies, i.e. subsequent testing of a reported gene association in a distinct population as was also clearly pointed out by Colhoun and colleagues[36].

Reference List

1. Nunes, H., Soler, P. & Valeyre, D. Pulmonary sarcoidosis. *Allergy. 2005 May* **60**, 565-582.
2. Reich, J. M. Mortality of intrathoracic sarcoidosis in referral vs population-based settings: influence of stage, ethnicity, and corticosteroid therapy. *Chest* -9 (2002).
3. Eishi, Y. *et al.* Quantitative analysis of mycobacterial and propionibacterial DNA in lymph nodes of Japanese and European patients with sarcoidosis. *J Clin Microbiol 2002 Jan;40(1):198-204.* -204.
4. Fite, E., Fernandez-Figueras, M. T., Prats, R., Vaquero, M. & Morera, J. High Prevalence of Mycobacterium tuberculosis DNA in Biopsies from Sarcoidosis Patients from Catalonia, Spain. *Respiration 2005 Aug 17;.*
5. du Bois, R. M., Goh, N., McGrath, D. & Cullinan, P. Is there a role for microorganisms in the pathogenesis of sarcoidosis? *J Intern Med. 2003 Jan* **253**, 4-17.
6. Takeda, K. & Akira, S. Toll-like receptors in innate immunity. *Int Immunol. 2005 Jan* **17**, 1-14.
7. Takeda, K. & Akira, S. Toll-like receptors in innate immunity. *Int Immunol. 2005 Jan* **17**, 1-14.
8. Pabst, S. *et al.* Toll-like receptor (TLR) 4 polymorphisms are associated with a chronic course of sarcoidosis. *Clin Exp Immunol. 2006 Mar* **143**, 420-426.
9. Arbour, N. C. *et al.* TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. *Nat Genet. 2000 Jun* **25**, 187-191.
10. Hunninghake, G. W. *et al.* ATS/ERS/WASOG statement on sarcoidosis. American Thoracic Society/European Respiratory Society/World Association of Sarcoidosis and other Granulomatous Disorders. *Sarcoidosis Vasc Diffuse Lung Dis 1999 Sep;16(2):149-73.* -73.
11. Van Rijn, B. B., Roest, M., Franx, A., Bruinse, H. W. & Voorbij, H. A. Single step high-throughput determination of Toll-like receptor 4 polymorphisms. *J Immunol Methods. 2004 Jun* **289**, 81-87.
12. Pabst, S. *et al.* Toll-like receptor (TLR) 4 polymorphisms are associated with a chronic course of sarcoidosis. *Clin Exp Immunol. 2006 Mar* **143**, 420-426.
13. Pabst, S. *et al.* Toll-like receptor (TLR) 4 polymorphisms are associated with a chronic course of sarcoidosis. *Clin Exp Immunol. 2006 Mar* **143**, 420-426.
14. Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J. & Mathison, J. C. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science. 1990 Sep* **21;249**, 1431-1433.
15. Hopkins, P. A. & Sriskandan, S. Mammalian Toll-like receptors: to immunity and beyond. *Clin Exp Immunol. 2005 Jun* **140**, 395-407.
16. Arbour, N. C. *et al.* TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. *Nat Genet. 2000 Jun* **25**, 187-191.

17. Imahara, S. D., Jelacic, S., Junker, C. E. & O'Keefe, G. E. The TLR4 +896 polymorphism is not associated with lipopolysaccharide hypo-responsiveness in leukocytes. *Genes Immun. 2005 Feb* **6**, 37-43.

18. van der, G. C. *et al.* Functional consequences of the Asp299Gly Toll-like receptor-4 polymorphism. *Cytokine. 2005 Jun* **7;30**, 264-268.

19. Takeda, K. & Akira, S. Toll-like receptors in innate immunity. *Int Immunol. 2005 Jan* **17**, 1-14.

20. Dubaniewicz, A., Kampfer, S. & Singh, M. Serum anti-mycobacterial heat shock proteins antibodies in sarcoidosis and tuberculosis. *Tuberculosis (Edinb). 2006 Jan* **86**, 60-67.

21. Pabst, S. *et al.* Toll-like receptor (TLR) 4 polymorphisms are associated with a chronic course of sarcoidosis. *Clin Exp Immunol. 2006 Mar* **143**, 420-426.

22. Lorenz, E., Frees, K. L. & Schwartz, D. A. Determination of the TLR4 genotype using allele-specific PCR. *Biotechniques. 2001 Jul* **31**, 22-24.

23. Van Rijn, B. B., Roest, M., Franx, A., Bruinse, H. W. & Voorbij, H. A. Single step high-throughput determination of Toll-like receptor 4 polymorphisms. *J Immunol Methods. 2004 Jun* **289**, 81-87.

24. Hang, J. *et al.* TLR4 Asp299Gly and Thr399Ile polymorphisms are very rare in the Chinese population. *J Endotoxin Res. 2004* **10**, 238-240.

25. Zee, R. Y., Hegener, H. H., Gould, J. & Ridker, P. M. Toll-like receptor 4 Asp299Gly gene polymorphism and risk of atherothrombosis. *Stroke. 2005 Jan* **36**, 154-157.

26. Cooke, G. S., Segal, S. & Hill, A. V. Toll-like receptor 4 polymorphisms and atherogenesis. *N Engl J Med. 2002 Dec* **12;347**, 1978-1980.

27. Netea, M. G. *et al.* Toll-like receptor-4 Asp299Gly polymorphism does not influence progression of atherosclerosis in patients with familial hypercholesterolaemia. *Eur J Clin Invest. 2004 Feb* **34**, 94-99.

28. Hawn, T. R. *et al.* Toll-like receptor 4 polymorphisms are associated with resistance to Legionnaires' disease. *Proc Natl Acad Sci U S A. 2005 Feb* **15;102**, 2487-2489.

29. Ouburg, S. *et al.* The toll-like receptor 4 (TLR4) Asp299Gly polymorphism is associated with colonic localisation of Crohn's disease without a major role for the Saccharomyces cerevisiae mannan-LBP-CD14-TLR4 pathway. *Gut. 2005 Mar* **54**, 439-440.

30. van der, P. M. *et al.* No evidence for involvement of the Toll-like receptor 4 (TLR4) A896G and CD14-C260T polymorphisms in susceptibility to ankylosing spondylitis. *Ann Rheum Dis. 2005 Feb* **64**, 235-238.

31. Pabst, S. *et al.* Toll-like receptor (TLR) 4 polymorphisms are associated with a chronic course of sarcoidosis. *Clin Exp Immunol. 2006 Mar* **143**, 420-426.

32. Kroner, A. *et al.* Impact of the Asp299Gly polymorphism in the toll-like receptor 4 (tlr-4) gene on disease course of multiple sclerosis. *J Neuroimmunol. 2005 Aug* **165**, 161-165.

33. Reismann, P. *et al.* Lack of association between polymorphisms of the toll-like receptor 4 gene and cerebral ischemia. *J Neurol. 2004 Jul* **251**, 853-858.

34. Brand, S. *et al.* The role of Toll-like receptor 4 Asp299Gly and Thr399Ile polymorphisms and CARD15/NOD2 mutations in the susceptibility and phenotype of Crohn's disease. *Inflamm Bowel Dis. 2005 Jul* **11**, 645-652.

35. Rohde, G. *et al.* Association of the ASP299GLY TLR4 polymorphism with COPD. *Respir Med. 2006 May* **100**, 892-896.

36. Colhoun, H. M., McKeigue, P. M. & Davey, S. G. Problems of reporting genetic associations with complex outcomes. *Lancet. 2003 Mar* **8;361**, 865-872.

3

Linkage between Toll-like receptor (TLR) 2 promoter and intron polymorphisms; functional effects and relevance to sarcoidosis

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Summary

The intracellular pathogens *Propionibacterium acnes* and *Mycobacterium tuberculosis* have been leading suspects as the cause of sarcoidosis, a systemic disorder characterized by the formation of non-caseating granulomas. Toll-like receptor (TLR) 2 is important in the innate immune response against both pathogens and therefore of interest in sarcoidosis research. In the present study three single nucleotide polymorphisms and one dinucleotide repeat polymorphism in the TLR-2 gene were genotyped in 419 sarcoidosis patients, divided in a study cohort and a validation cohort, and 196 healthy controls. In the study cohort we found a significant increase in prevalence of the AA-genotype at promoter location –16934 in patients with chronic disease compared to patients with acute/self remitting sarcoidosis (34.5% vs. 15.9% respectively, $p=0.006$, $p_c=0.019$). These results could not be confirmed in our validation cohort, implicating a possible role for TLR-2 genetics in only a small percentage of sarcoidosis patients. Furthermore, linkage was found between the promoter polymorphism –16934 A/T and the number of GT-repeats in intron 1 ($p<0.0001$). After in vitro stimulation of Peripheral Blood Mononuclear Cells (PMBCs) with different TLR-2 agonists, a correlation between induction of TNF-α ($p=0.008$), IL-12 ($p=0.008$) as well as IL-6 ($p=0.02$) and the number of GT-repeats was observed. In conclusion, the data show that polymorphisms in TLR-2 might be important in a small group of sarcoidosis patients and that their functional consequences partly explain some of the variance in cytokine pattern observed in different clinical phenotypes of this disease.

Introduction

Sarcoidosis is a systemic disorder characterized by the formation of non-caseating granulomas, most often affecting the lungs, lymph nodes and skin. The etiology remains unknown, but it is attractive to speculate, given the variation in the clinical spectrum of sarcoidosis, that this disease reflects a collection of different granulomatous diseases, each with its own antigen. Sarcoidosis is characterized by a strong cell-mediated immune reaction, making pathogens such as viruses or intracellular bacteria the leading suspects as causative agents. Especially *Propionibacterium acnes* and *Mycobacterium tuberculosis* have been extensively studied and may well play a role in disease pathogenesis[1-5].

Recent years have seen a dramatic improvement of our understanding of the role of innate immunity in human host defence. Innate immunity is able to discriminate between self and a variety of pathogens and can initiate inflammatory and anti-microbial responses, thereby dictating the ensuing adaptive immune response. To date, relatively little attention has been paid to the role of innate immune receptors in the pathogenesis of sarcoidosis. Single nucleotide polymorphisms (SNPs) in genes encoding for Mannose Binding Lectin (MBL2) and the Nucleotide-binding Oligomerization domains (NOD) 1 and 2 have been studied in sarcoidosis patients, showing that only NOD1 is likely to play a role in disease pathogenesis[6-9]. Regarding CD14, the classical LPS receptor, it has been shown that the concentration of its soluble form (sCD14) in Bronchoalveolar Lavage (BAL) fluid is higher in patients with active sarcoidosis compared with inactive sarcoidosis[10]. Furthermore, Gazouli *et al.* demonstrated that the CD14 promoter polymorphism –159C/T is associated with sarcoidosis patients in Greece[11]. These studies suggest that an altered regulation of the innate immune response can play a role in sarcoidosis pathogenesis, which already has been demonstrated for other granulomatous disorders like Crohn's disease and tuberculosis [12-15].

An important group of innate immunity receptors is the family of Toll-like receptors (TLRs), consisting of 10 receptors, each recognizing a distinct, but limited, repertoire of microbial products. At present, 3 genetic studies have been published addressing the role of this receptor family in sarcoidosis. The influence of TLR-4 polymorphisms

on the disease course of sarcoidosis patients has been studied by several research groups, including ours, but with conflicting results[16-18].

Taken together, when considering a causative role for intracellular bacteria in the pathogenesis of sarcoidosis and the possible influence of altered innate immunity, TLRs recognizing microbial components of *P. acnes* or *M. tuberculosis* are of interest. TLR-2 is involved in the immune response against both pathogens and seems a primary candidate for genetic and functional analysis[19-21]. TLR-2 signalling also seems important for the function of regulatory T cells, a population of T cells which might be functionally impaired in sarcoidosis [22,23]. We hypothesized that an aberrant innate immune response against TLR-2 agonists, such as *P. acnes* or *M. tuberculosis,* contributes to the development of sarcoidosis, or at least influences disease course. In the present study we tested the hypothesis that genetic variances altering TLR-2 function are more prevalent in (subgroups of) sarcoidosis patients.

Materials and Methods

Subjects

One hundred sixty-five unrelated and randomly selected Dutch Caucasian patients with sarcoidosis (92 men and 73 women) were included in the study cohort. In 108 patients, the diagnosis of sarcoidosis was established when clinical findings were supported by histological evidence, and after the exclusion of other known causes of granulomatosis. 57 patients presented with the classical Löfgren's syndrome of fever, erythema nodosum, bilateral hilar lymphadenopathy and joint symptoms. Verbal and written consent was obtained from all subjects, and authorization was given by the Ethics Committee of the St. Antonius Hospital, Nieuwegein. The control subjects comprised 196 healthy Dutch Caucasian employees of the St. Antonius Hospital in The Netherlands. By completing a questionnaire, relevant background information was provided by these volunteers and included medication, ethnicity and hereditary diseases. As a validation cohort, 254 unrelated and randomly selected Caucasian patients with sarcoidosis from a hospital located in a different geographic region in The Netherlands were used. Sarcoidosis was diagnosed as

described above and written consent was obtained from these subjects as well as approval by the Ethics Committee of the University Hospital, Maastricht.

Evaluation of pulmonary disease severity

Chest radiographs at presentation, 2 years and 4 years were collected for each patient and assessed blind by a pulmonary physician for disease severity using standard radiographic staging for sarcoidosis. In brief, this comprises five stages: stage 0 = normal, stage I = bilateral hilar lymphadenopathy (BHL), stage II = BHL and parenchymal infiltration, stage III = parenchymal infiltration without BHL, stage IV = irreversible fibrosis with loss of lung volume. Radiographic evolution over a 4 year period was available for 165 patients and was categorized as follows; A: normalization of improvement towards stage I (n=50), B: persistent stage II/III or progression in that direction (n=35), and C: stable stage IV or progression towards this stage (n=23) [24]. Patients who had been diagnosed with Löfgren's syndrome at presentation (n= 57) were considered as a distinct group with radiographic evolution not exceeding stage I. In the validation cohort there were 42 patients with Löfgren's syndrome, 72 patients were categorized as stage A, 105 patients as stage B and 35 patients as stage C. Patients with Lögren's syndrome or stage A were defined as acute/self-remitting disease and patients with stages B or C as chronic disease.

Analysis of TLR-2 genetic single nucleotide polymorphisms

Genomic DNA extracted from all subjects and controls was genotyped for three different TLR-2 (SNPs) using sequence-specific primers (SSPs) and polymerase chain reaction, or Taqman genotyping assay when SSP-PCR was technically impossible (16934A/T). Three known SNPs and one short tandem repeat polymorphism in the TLR-2 gene were selected based on associations with gram-positive infections and Tuberculosis[25,26]. The 3 SNPs were located at the following nucleotide positions (including NCBI reference numbers): -16934 (rs4696480, promoter region) +1892 (rs5743704/Pro^{631}His, exon 3) and +2258 (rs5743708/Arg^{753}Gln, exon 3). Identification of the –16934A/T polymorphism was performed using the dual labeled allele specific oligonucleotides 5'-[FAM]-ACCCTCTCCAGATGC-[NFQ] and 5'-[VIC™]

CCCT CACCAGATGC-[NFQ] together with forward primer 5′-GGTTCTGGAGTCTG GGAAGTC and reverse primer 5′-TCTCACCATGTGATGCTTTCCATC following manufacturer's instructions (Custom TaqMan® SNP Genotyping Assay, Applied Biosystems). For the identification of the polymorphism at position +1892 the sequence-specific reverse primers 5′-CTGCTGGGAGCTTTCCTGG and 5′-CTGC TGGGAGCTTTCCTGT were combined with the forward primer 5′-AGCAAG CACTGGCCAAAGTCT leading to an expected PCR product size of 268 bp. For identification of the polymorphism at position +2258 the sequence-specific reverse primers 5′-AGGTCTTGGTGTTCATTATCTTCT and 5′-AGGTCTTGGTGTTC ATTATCTTCC were combined with the forward primer 5′-AGCAAGCACTGGC CAAAGTCT leading to an expected PCR product size of 406 bp. In all primer mixes we included control primers 5-ATGATGTTGACCTTTCCAGGG and 5′-GCAACT GATGAAAAGTTACAGAA leading to an expected PCR product size of 720bp. All PCR reactions were run under identical conditions as previously described [27].

Analysis of TLR-2 intron 1 microsatellite polymorphism

The TLR-2 GT dinucleotide repeat is located 100 bp upstream of the translational start site in intron 1 (http://SNPper.chip.org). A region of about 150bp encompassing this repeat polymorphism was amplified using the primers 5′-ATGATATATATAG ATAT, labeled with FAM and primer 5′-ATATAGAGAGAGTA [28]. The PCR reactions were run as previously described [29]. Gene Scan was performed on an ABI PRISM® 3100-Avant Genetic Analyzer (Applied Biosystems) and results were analyzed with Gene Mapper™ 3.5 (Applied Biosystems).

TLR-2 surface expression

The following monoclonal antibodies (mAbs) were used in the flow cytometric studies: anti-CD45 (PerCP) , anti-CD3 (Fitc) and anti-CD14 (APC) all from BD Biosciences. Anti-TLR-2 (PE, clone TL2.1) was purchased from eBioscience. Fifty µl of EDTA-blood was incubated for 15 min. at room temperature with 10µl of the following mAbs: anti-CD45, anti-CD3, anti-CD14 and anti-TLR-2. The CD14+ population was gated after selecting the CD45+ population. The granulocyte population was selected based on FSC-SSC characteristics. Immunofluoresence was measured by flowcytometry (FACSCalibur, Becton Dickinson) and data was analyzed with CellQuest software

(BD Biosciences). During each experiment a total of 20.000 cells were counted and experiments were performed in triplicate.

Quantification of cytokine production

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood using Ficoll-Paque density gradient centrifugation and seeded on 24-well plates at a density of 400.000 cells per well. PBMCs were stimulated with the TLR-2 agonist Pam_3Cys-Ser-Lys_4 (10µg, EMC Microcollections) or with clinical isolates of either *Propionibacterium acnes* or *Mycobacterium tuberculosis* (100.000/well). *P. acnes* was identified based on morphology, gram staining and both indolase and catalase producing characteristics and 2 different clinical isolates were pooled. Determination of *M. tuberculosis* was performed as previously described[30]. Prior to the experiments, *M. tuberculosis* was heat-inactivated at 100°C for 30 minutes. Phytohemagglutinin (42µg, Murex) and medium were used as positive and negative control, respectively. All stimulations were performed in a final volume of 1200µl RPMI 1640 (Gibco) containing 10% heat-inactivated FCS (Gibco) and 1% Clindamycin/Streptomycin. After a 24-hrs incubation period at 37°C in humidified air containing 5% CO_2, supernatants were collected and immediately stored at -80°C until further analysis. Five different cytokines (IL-12p70, IFN-γ, TNF-α, IL-4 and IL-6) were measured using Multiplex Immuno Assay (Biorad) on a Bioplex 100 system (Biorad) according to the manufacturer's instructions. Calibration curves were constructed using the medium mentioned above. For the samples with IL-6 concentrations above the upper detection limit, IL-6 was determined in serial dilutions of culture supernatant by sandwich ELISA (Sanquin, Amsterdam, The Netherlands).

Statistical analysis

Allele and genotype frequencies were calculated for each locus and tested for Hardy-Weinberg equilibrium in controls. Association studies were analysed by chi-square testing using 2x3 and 2x2 contingency tables for genotype and allele frequencies, respectively. Data analysis was carried out using the Student's *t*-test. Correlation analysis was performed using a Pearson test. P-values were corrected using a Bonferroni correction for multiple testing. A p value <0.05 was considered significant.

Results

Allelic distribution of TLR-2 single nucleotide polymorphisms and intron repeat polymorphism in patients and controls

The genotype distribution of the investigated TLR-2 SNPs in our study cohort and controls are presented in table 1. Genotype data of the healthy controls conformed to Hardy-Weinberg equilibrium.

Genotype		Controls (n=196)	Patients (n=165)	LOF (n=57)	A* (n=50)	B* (n=35)	C* (n=23)
-16934	TT	47	43	20	12	7	4
	TA	102	85	29	29	15	12
	AA	47	37	8	9	13	7
$Pro^{631}His$	CC	181	151	55	46	28	22
	CA	15	14	2	4	7	1
$Arg^{753}Trp$	GG	185	155	55	47	33	20
	GA	11	10	2	3	2	3

Table 1. Genotype distribution of the TLR-2 single nucleotide polymorphisms in sarcoidosis patients from the study cohort and healthy controls. LOF = patients with Löfgren's syndrome. * indicates radiographic evolution according to the Scadding criteria over a minimal 4 year period. A = normalization of improvement towards stage I. B = persistent stage II/III or progression in that direction and C = stable stage IV or progression towards this stage.

When assigning all sarcoidosis patients to one group, no differences were found between patients and controls regarding the allelic distribution of the 3 SNPs and the number of GT tandem repeats. However, comparison of patients with acute/ self-remitting disease with patients having chronic disease revealed that -16934 T allelic carriage was more prevalent in the first group (84.1% vs. 65.5%, respectively, p=0.006, p_c=0.019, figure 1). Furthermore, we found that the distribution of the number of GT intron repeats also differed between patients with acute/self remitting disease and patients having chronic disease (figure 2), with a higher prevalence of relative short alleles in the latter group.

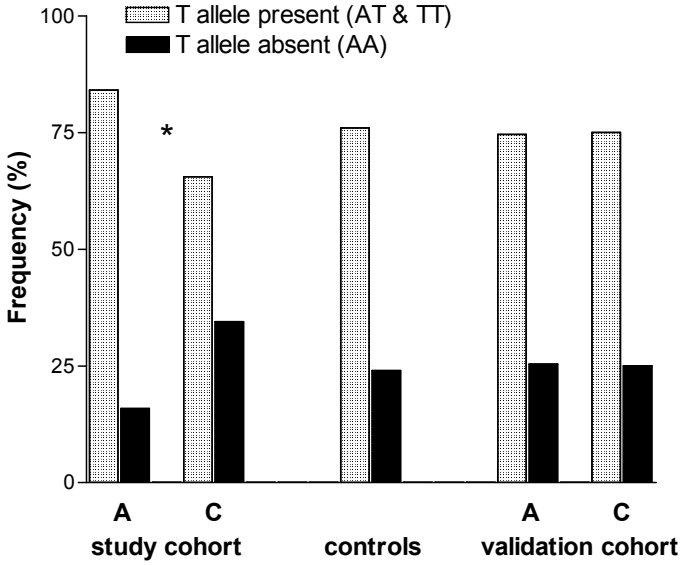

Figure 1. TLR-2 promoter polymorphism –16934 T allele carriage in sarcoidosis patients with acute/self remitting disease vs chronic disease. **A** = acute/self-remitting disease, **C** = chronic disease. * indicates p_c = 0.019 (uncorrected p value = 0.006).

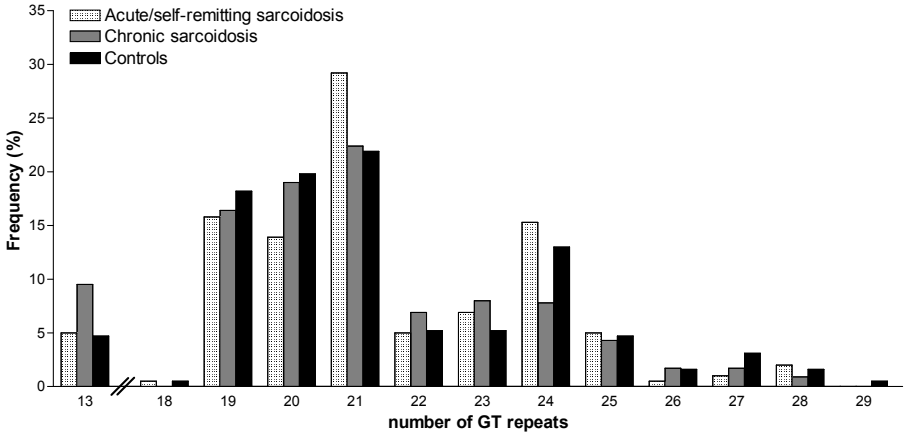

Figure 2. Allelic distribution of TLR-2 intron 1 GT-repeat polymorphism in sarcoidosis patients from the study cohort and healthy controls.

To confirm our genetic association we determined the –16934 T allele carriage in a validation cohort consisting of 254 unrelated and randomly selected Caucasian patients with sarcoidosis from a separate geographic region within The Netherlands. In this cohort we did not find a difference in T allele carriage between patients with acute/self-remitting disease and chronic disease (acute/self-remitting: AA n=29, AT n=56, TT n=29, chronic: AA n=35, AT n= 69, TT n= 36, p=0.92).

Linkage between TLR-2 promoter polymorphism –16934A/T and intron 1 GT-repeat polymorphism

The distribution of the promoter polymorphism and the intron 1 repeat polymorphism differed between patients with an acute/self remitting form of sarcoidosis and patients having a chronic disease form in the study cohort. Because single nucleotide polymorphisms and short tandem repeats occur frequently in the genome, closely linked pairs are common. We therefore tested whether these two polymorphisms were in linkage disequilibrium. Both polymorphisms were determined in 96 healthy Dutch controls. We found a highly significant association between the TLR-2 promoter polymorphism and the number of tandem repeats in the intron 1 polymorphism (figure 3). The A allele was mainly linked to GT-repeat numbers 13,19,20 and 21, the T allele was mainly linked to repeat numbers 21-27. The mean number of GT tandem repeats was 19.5 in the AA-genotype group, 21.3 in the AT-genotype group and 23.1 in the TT-genotype group ($p<0.0001$). A similar association was also found in sarcoidosis patients (data not shown, $p<0.0001$).

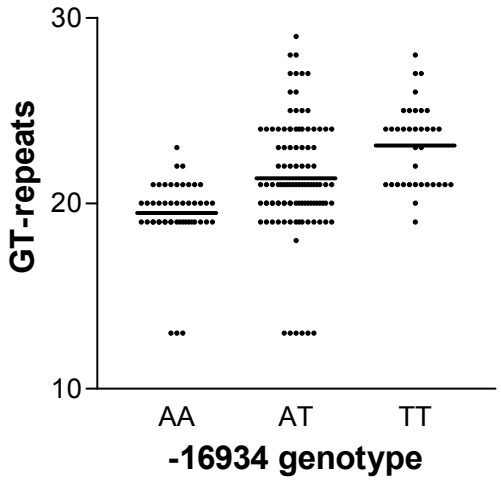

Figure 3. Linkage between the genotype of TLR-2 promoter polymorphism -16934A/T and the number of intron 1 GT-repeats. 192 GT-alleles derived from 96 healthy donors are grouped according to –16934 genotype. Horizontal bars indicate the mean number of GT-repeats. The mean number of GT tandem repeats was 19.5 in the AA-genotype group, 21.3 in the AT-genotype group and 23.1 in the TT-genotype group ($p<0.0001$).

Influence of TLR-2 promoter polymorphism –16934A/T and intron 1 GT-repeat polymorphism on TLR-2 expression and cytokine production

Considering the discrepant genetic results on the distribution of promoter polymorphism –16934A/T and intron 1 repeat polymorphism in two independent groups of sarcoidosis patients we decided to investigate the functional relevance of both polymorphisms. First, we measured basal TLR-2 expression on both granulocytes and CD14 positive monocytes in 15 healthy donors with either promoter genotype AA (n=5), AT (n=5) or TT (n=5) and a corresponding variable number of GT-repeats. We did not find a correlation between neither of the two polymorphisms and TLR-2 expression on granulocytes or monocytes (figures 4 & 5).

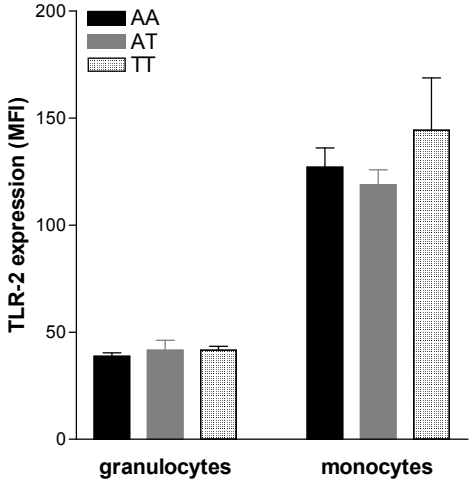

Figure 4. Influence of the TLR-2 promoter polymorphism –16934A/T on the TLR-2 cell surface expression measured on both granulocytes and monocytes. No differences were found between TLR-2 expression and different promoter genotypes (granulocytes; p=0.77, monocytes; p=0.52). MFI = Mean Fluoresence Intensity (+SEM).

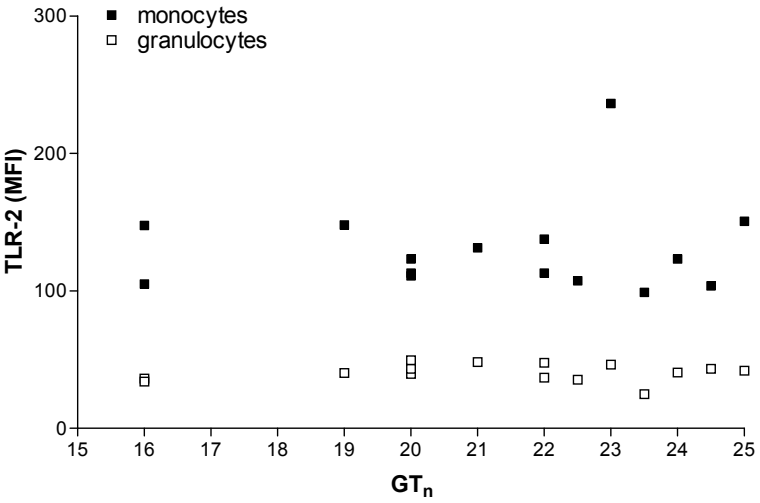

Figure 5. Influence of the number of TLR-2 intron 1 GT-repeats on TLR-2 surface expression measured on both granulocytes and monocytes. No correlation was found between TLR-2 expression and the number of GT-repeats (granulocytes p=0.70, monocytes p=0.78). MFI = Mean Fluoresence Intensity. GT_n = mean number of GT repeats per donor.

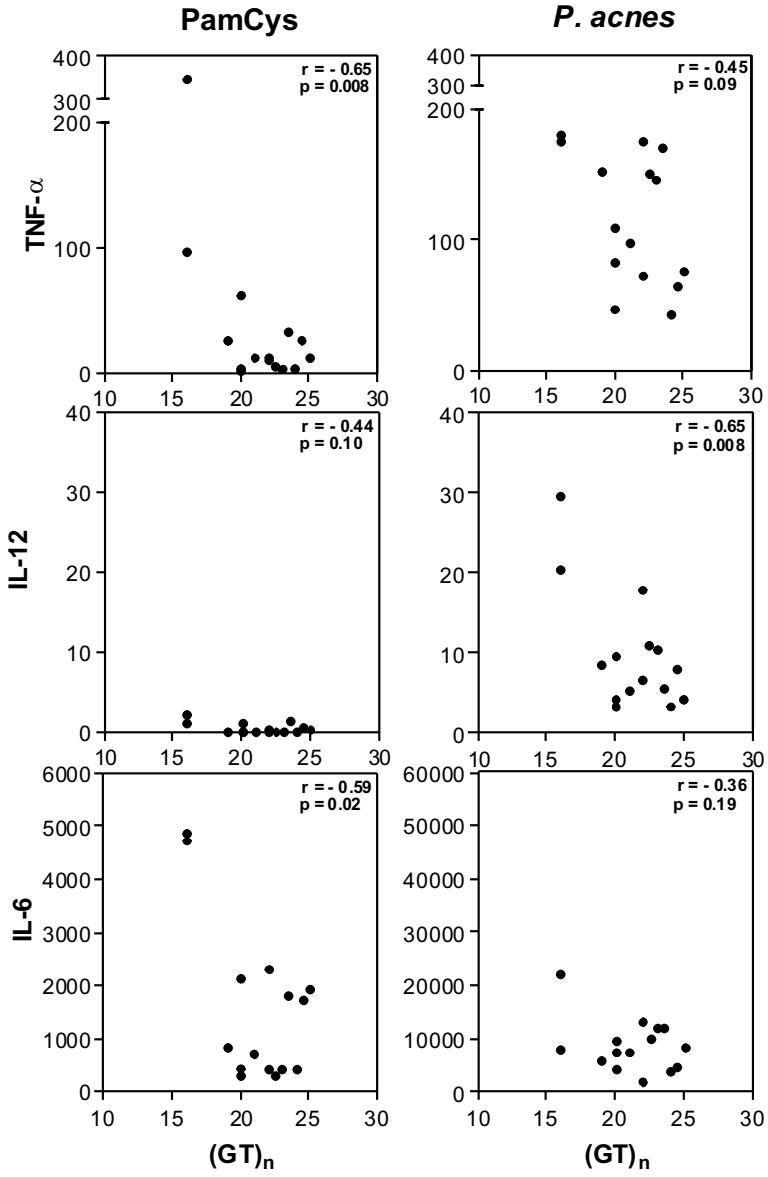

Figure 6. Influence of the number of TLR-2 intron 1 GT repeats on the induction of pro-inflammatory cytokines (in pg/ml) after stimulation of PBMCs with different TLR-2 agonists. PamCys = Pam_3Cys-Ser-$(Lys)_4$, *P. acnes* = *Propionibacterium acnes*. TNF-a = Tumor Necrosis Factor –alpha, IL-6 = Interleukin 6, IL-12 = Interleukin 12. GT_n = mean number of GT repeats per donor.

Secondly, production of pro-inflammatory cytokines was studied in PMBCs from the same 15 healthy donors after stimulation with different TLR-2 agonists (Pam_3Cys-Ser-Lys_4, *P. acnes* and *M. tuberculosis*). We observed a correlation between the production of TNF-α, IL-12 as well as IL-6 and the number of GT-repeats after stimulation with PamCys or *P. acnes* (figure 6). The effect of TLR-2 polymorphisms on production of pro-inflammatory cytokines was also found when data were grouped according to -16934 T allele carriage (figure 7). No correlation was found between IFN-γ or IL-4 and the number of GT-repeats (data not shown). Also, as expected, no correlations were found after stimulation with Phytohaemagglutinin (PHA). The use of heat-killed *M. tuberculosis* lead to a small, insignificant induction of cytokines (data not shown).

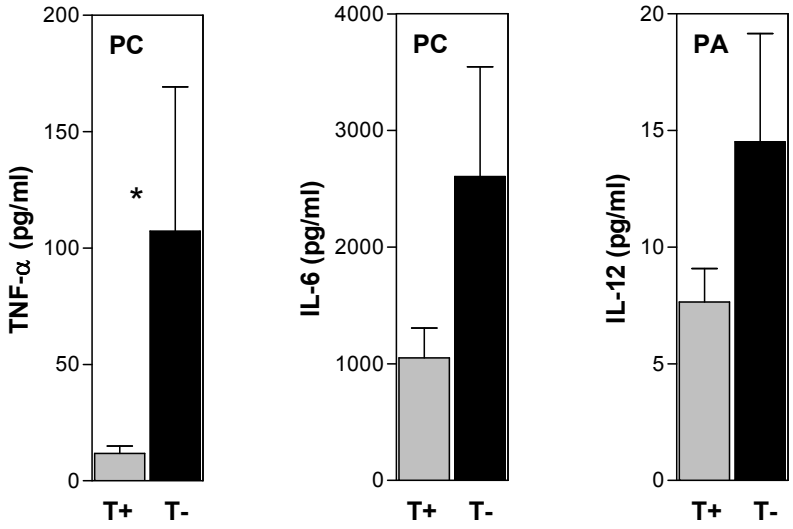

Figure 7. Influence of the TLR-2 –16934 promoter T allele carriage on the induction of pro-inflammatory cytokines after stimulation of PBMCs with different TLR-2 agonists. T+ = T allele present, T- = T allele absent. PC = stimulated with Pam_3Cys-Ser-Lys_4, PA = stimulated with *Propionibacterium acnes*. TNF-α = Tumor Necrosis Factor –alpha, IL-12 = Interleukin 12, IL-6 = Interleukin 6. Values are in picogram per milliliter and depicted +SEM. * indicates $p<0.05$.

Discussion

In the present study we investigated the genetic influence of the innate immunity receptor TLR-2 in Dutch sarcoidosis patients. TLR-2 is primarily expressed on monocytes, macrophages, dendritic cells, B cells and, to a lesser extent, on neutrophils[31]. Activation of TLR-2 results in the production of pro-inflammatory cytokines and up-regulation of co-stimulatory molecules and antigen presentation, thereby leading to priming of the ensuing adaptive immune response[32]. TLR-2 recognizes microbial components of *Propionibacterium acnes* and *Mycobacterium tuberculosis*, both possible causative agents for sarcoidosis, and is therefore of interest when postulating a role for an altered innate immune response in the pathogenesis of this disease.

The results did not show an association between TLR-2 polymorphisms Pro^{631}His or Arg^{753}Gln and disease susceptibility or influence on disease course in Dutch sarcoidosis patients. A major role for these functional polymorphisms therefore seems unlikely, however, in order to definitely exclude a role for these genetic variants, studies including greater number of patients together with cohorts of non-Caucasian ethnicity are required. Pertaining to polymorphism –16934A/T we found a significantly higher prevalence of the –16934-AA genotype in patients with chronic disease compared to patients with acute/self remitting disease in the study cohort, partially confirming our hypothesis. To establish the importance of this genetic association, an unrelated validation cohort of 254 Dutch sarcoidosis patients was also genotyped. The allelic distribution of this polymorphism in both patients with acute/self-remitting disease and chronic disease were identical and comparable with the healthy controls (figure 1). These results make it unlikely that TLR-2 genetics plays a major role in sarcoidosis pathogenesis. On the other hand however, assuming that sarcoidosis has multiple causative agents, it could be that TLR-2 agonists are triggering agents in only a small fraction of the entire population of Dutch patients, making it difficult to compare results from small independent patients groups. An elegant way to overcome this problem could be to subdivide patients by causative agents. In light of the cellular recognition of *M. tuberculosis* and *P.acnes* antigens in some sarcoidosis patients[33,34], future studies involving TLR-2 genetics in these particular subgroups should reveal more accurate data.

The present study also revealed a strong linkage between the promoter polymorphism and the GT-repeat polymorphism in intron 1. Based on this linkage disequilibrium, the difference in allelic distribution of the GT-repeat polymorphism between acute/self-remitting and chronic sarcoidosis found in the study cohort could not be confirmed in the validation cohort. The allelic distribution found in the control population was almost identical with the distribution found in a Caucasian-American cohort [35], reflecting the reliability of our method. The linkage between the TLR-2 promoter polymorphism –16934A/T and the intron 1 GT-repeat polymorphism is of interest not only in sarcoidosis research, but for all research focussing on one of these polymorphisms. A major question is to what extent do these two polymorphisms have functional implications on TLR-2 receptor expression and function? So far, no data are available on the functional implications of the –16934A/T promoter polymorphism. Two papers regarding the influence of the number of GT- intron repeats on TLR-2 expression from the same group have been published, demonstrating strong conflicting results[36,37]. Based on the discrepant genetic data on the distribution of TLR-2 polymorphisms in 2 different cohorts of Dutch sarcoidosis patients and the indistinctness regarding their functional implications we investigated a possible influence on TLR-2 expression and function. After stimulation with PamCys, the most specific TLR-2 agonist, we found a correlation between TNF-α production as well as IL-6 production and the number of GT-repeats. After stimulation with *P.acnes*, which stimulates not only TLR-2 but also many other innate immune receptors, the same trend could still be observed regarding TNF-α production. Furthermore, stimulation with PamCys resulted in limited amounts of IL-12 production related to the number of GT-repeats, although not statistically significant. *P. acnes* however, is a more potent inducer of IL-12 compared with the peptide PamCys, and with this stimulus a significant correlation between IL-12 production and the number of GT-repeats was found. We did not find a correlation between the number of GT-repeats and the production of prototype T-helper-1 (IFN-γ) or T-helper-2 (IL-4) cytokines. Toll-like receptors are predominantly associated with the production of pro-inflammatory cytokines, possibly explaining the absence of a correlation between production of specific T-helper cell associated cytokines and number of GT-repeats.

The number of GT-repeats is not the only genetic variant in TLR-2 that influences cytokine production upon stimulation. Underhill and colleagues showed that the presence of TLR-2 polymorphism Pro[631]His results in a 5-fold reduction of TNF-α production upon stimulation with *M. tuberculosis*[38]. Although using PamCys instead of *M. tuberculosis*, when we grouped our data according to –16934 T allele carriage we found a 9-fold increase of TNF-α production in the group without a T allele (figure 7), indicating the importance of our findings.

An obvious mechanism of how a variable number of GT-repeats might affect TLR-2 function remains unknown. In our functional analysis we used the mean number of GT-repeats per donor based on the assumption that possible influences on TLR-2 function by a variable number of GT-repeats would follow a linear relation. However, it remains to be seen if the impact of an allele with a small number of GT-repeats in combination with an allele with a large number of GT-repeats equals that of a combination consisting of 2 "medium-sized GT-alleles". For example, when the induced amounts of TNF-α, IL-12 or IL-6 were plotted against the presence of either the allele with the smallest number of GT-repeats per donor or the allele containing the largest number of GT-repeats per donor, we only found correlations in the first group (figure 8). The observed correlations using the smallest alleles proved to be stronger than the correlations using the mean number of GT-repeats. This might be an indication that the presence of a small number of GT-repeats could be dominant over the presence of a large number of GT-repeats, presenting an interesting viewpoint for further studies.

The genetic data from the study cohort together with our functional data on cytokine induction fit in the current model of sarcoidosis pathogenesis. TNF-α is important in the initiation and perpetuation of inflammation in sarcoidosis, contributing to progressive disease with or without development of fibrosis. Various clinical trials demonstrated that blocking TNF-α using antagonists such as infliximab or etanercept[39] results in clinical improvement and reduces the requirement of corticosteroids. The amount of IL-12, the main macrophage derived molecule involved in initiating a T-helper-1 response, and IL-6, known for making T cells insensitive for suppression by regulatory T cells[40], are also correlated with persisting disease activity[41-44].

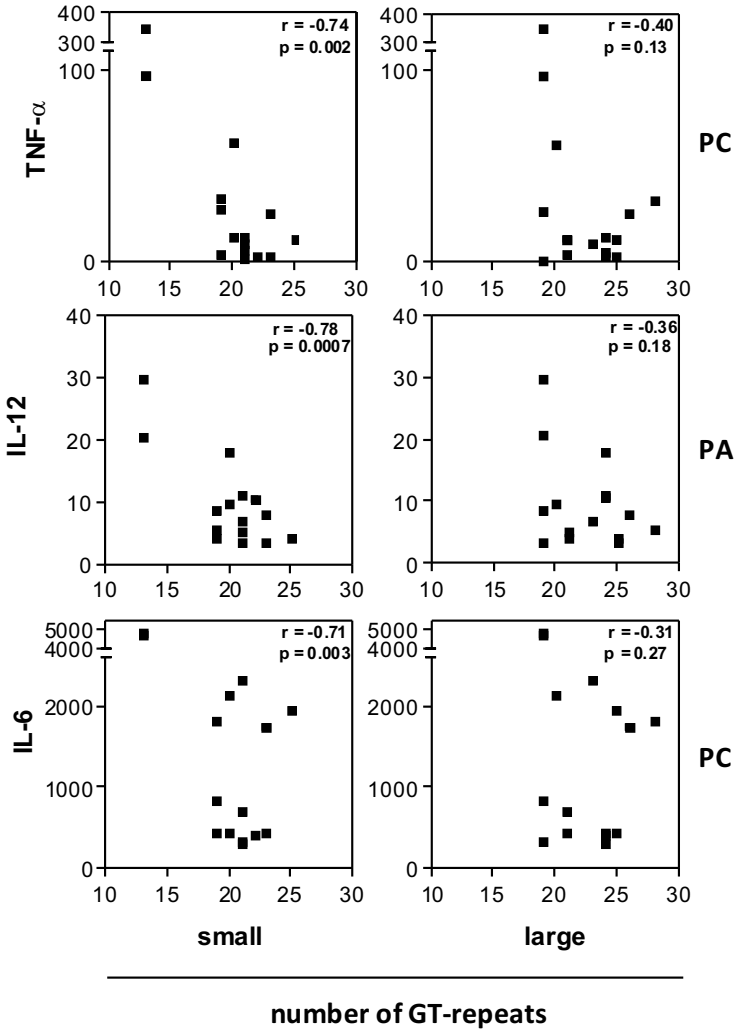

Figure 8. Observed differences in correlation between the smallest and the largest number of TLR-2 intron 1 GT-repeats per donor and cytokine induction (pg/ml) after stimulation with TLR-2 agonists. PC = stimulated with $Pam_3Cys\text{-}Ser\text{-}Lys_4$, PA = stimulated with *Propionibacterium acnes*. TNF-a = Tumor Necrosis Factor –alpha, IL-12 = Interleukin 12, IL-6 = Interleukin 6. Small = per donor the smallest number of GT-repeats present was selected for analysis. Large = per donor the largest number of GT-repeats present was selected for analysis.

In our study cohort we found that in patients with chronic disease the prevalence of the TLR-2 –16934 AA-genotype was significantly higher compared to patients with acute/self-remitting disease. It was also demonstrated that the AA-genotype is linked to a significant lower amount of TLR-2 intron GT-repeats, implicating a higher amount of TNF-α, IL-6 and IL-12 production upon stimulation with TLR-2 agonists (figure 7). We speculate that patients with the -16934 AA-genotype produce higher amounts of these cytokines during continuous innate immunity stimulation by a persisting antigen, compared to patients with either the AT- or TT-genotype, thereby increasing their risk of developing chronic disease.

In summary, we found a difference in allelic distribution of a single nucleotide polymorphism in the promoter region of TLR-2 between sarcoidosis patients with acute/self-remitting disease and patients with chronic disease. However, this genetic association could not be confirmed in an independent cohort of sarcoidosis patients, suggesting a possible role in only a small percentage of patients. Furthermore, this promoter polymorphism is linked with the number of GT-repeats in intron 1. These two polymorphisms appear to influence the induction of TNF-α, IL-6 and IL-12 upon interaction with TLR-2 agonists, partly explaining the variation in cytokine pattern observed in different sarcoidosis phenotypes. Further genetic as well as functional studies are therefore required to confirm and explain these observations.

Reference List

1. Dubaniewicz, A., Kampfer, S. & Singh, M. Serum anti-mycobacterial heat shock proteins antibodies in sarcoidosis and tuberculosis. *Tuberculosis (Edinb). 2006 Jan* **86**, 60-67.
2. Fite, E., Fernandez-Figueras, M. T., Prats, R., Vaquero, M. & Morera, J. High prevalence of Mycobacterium tuberculosis DNA in biopsies from sarcoidosis patients from Catalonia, Spain. *Respiration. 2006* **73**, 20-26.
3. Gazouli, M. *et al.* Assessment of mycobacterial, propionibacterial, and human herpesvirus 8 DNA in tissues of greek patients with sarcoidosis. *J Clin Microbiol. 2002 Aug* **40**, 3060-3063.
4. Song, Z. *et al.* Mycobacterial catalase-peroxidase is a tissue antigen and target of the adaptive immune response in systemic sarcoidosis. *J Exp Med. 2005 Mar* **7;201**, *755-767*.
5. Eishi, Y. *et al.* Quantitative analysis of mycobacterial and propionibacterial DNA in lymph nodes of Japanese and European patients with sarcoidosis. *J Clin Microbiol 2002 Jan;40(1):198-204.* -204.
6. Foley, P. J. *et al.* Mannose-binding lectin promoter and structural gene variants in sarcoidosis. *Eur J Clin Invest. 2000 Jun* **30**, 549-552.
7. Ho, L. P. *et al.* CARD 15 gene mutations in sarcoidosis. *Thorax. 2005 Apr* **60**, 354-355.
8. Milman, N., Nielsen, O. H., Hviid, T. V. & Fenger, K. CARD15 Single Nucleotide Polymorphisms 8, 12 and 13 Are Not Increased in Ethnic Danes with Sarcoidosis. *Respiration. 2006 Jan* **2;..**.
9. Tanabe, T. *et al.* Sarcoidosis and NOD1 variation with impaired recognition of intracellular Propionibacterium acnes. *Biochim Biophys Acta. 2006 Jul* **25;..**.
10. Striz, I. *et al.* Soluble CD14 is increased in bronchoalveolar lavage of active sarcoidosis and correlates with alveolar macrophage membrane-bound CD14. *Am J Respir Crit Care Med. 1995 Feb* **151**, *544-547*.
11. Gazouli, M. *et al.* CARD15/NOD2, CD14, and toll-like receptor 4 gene polymorphisms in Greek patients with sarcoidosis. *Sarcoidosis Vasc Diffuse Lung Dis. 2006 Mar* **23**, 23-29.
12. Marks, D. J. *et al.* Defective acute inflammation in Crohn's disease: a clinical investigation. *Lancet. 2006 Feb* **25;367**, 668-678.
13. Bafica, A. *et al.* TLR9 regulates Th1 responses and cooperates with TLR2 in mediating optimal resistance to Mycobacterium tuberculosis. *J Exp Med. 2005 Dec* **19;202**, 1715-1724.
14. Ogus, A. C. *et al.* The Arg753GLn polymorphism of the human toll-like receptor 2 gene in tuberculosis disease. *Eur Respir J. 2004 Feb* **23**, 219-223.
15. Yim, J. J. *et al.* The association between microsatellite polymorphisms in intron II of the human Toll-like receptor 2 gene and tuberculosis among Koreans. *Genes Immun. 2006 Mar* **7**, 150-155.

16. Pabst, S. *et al.* Toll-like receptor (TLR) 4 polymorphisms are associated with a chronic course of sarcoidosis. *Clin Exp Immunol. 2006 Mar* **143**, 420-426.

17. Veltkamp, M., Grutters, J. C., van Moorsel, C. H., Ruven, H. J. & van den Bosch, J. M. Toll-like receptor (TLR) 4 polymorphism Asp299Gly is not associated with disease course in Dutch sarcoidosis patients. *Clin Exp Immunol. 2006 Aug* **145**, 215-218.

18. Gazouli, M. *et al.* CARD15/NOD2, CD14, and toll-like receptor 4 gene polymorphisms in Greek patients with sarcoidosis. *Sarcoidosis Vasc Diffuse Lung Dis. 2006 Mar* **23**, 23-29.

19. Bafica, A. *et al.* TLR9 regulates Th1 responses and cooperates with TLR2 in mediating optimal resistance to Mycobacterium tuberculosis. *J Exp Med. 2005 Dec* **19;202**, 1715-1724.

20. Romics, L., Jr. *et al.* Toll-like receptor 2 mediates inflammatory cytokine induction but not sensitization for liver injury by Propioni- bacterium acnes. *J Leukoc Biol. 2005 Dec* **78**, 1255-1264.

21. Yim, J. J. *et al.* The association between microsatellite polymorphisms in intron II of the human Toll-like receptor 2 gene and tuberculosis among Koreans. *Genes Immun. 2006 Mar* ***7***, 150-155.

22. Liu, H., Komai-Koma, M., Xu, D. & Liew, F. Y. Toll-like receptor 2 signaling modulates the functions of CD4+ CD25+ regulatory T cells. *Proc Natl Acad Sci U S A. 2006 May* **2;103**, 7048-7053.

23. Miyara, M. *et al.* The immune paradox of sarcoidosis and regulatory T cells. *J Exp Med. 2006 Feb* **20;203**, 359-370.

24. Grutters, J. C. *et al.* Analysis of IL6 and IL1A gene polymorphisms in UK and Dutch patients with sarcoidosis. *Sarcoidosis Vasc Diffuse Lung Dis 2003 Mar;20(1):20-7. -7*.

25. Ogus, A. C. *et al.* The Arg753GLn polymorphism of the human toll-like receptor 2 gene in tuberculosis disease. *Eur Respir J. 2004 Feb* **23**, 219-223.

26. Underhill, D. M., Ozinsky, A., Smith, K. D. & Aderem, A. Toll-like receptor-2 mediates mycobacteria-induced proinflammatory signaling in macrophages. *Proc Natl Acad Sci U S A. 1999 Dec* ***7;96***, 14459-14463.

27. Bunce, M. *et al.* Phototyping: comprehensive DNA typing for HLA-A, B, C, DRB1, DRB3, DRB4, DRB5 & DQB1 by PCR with 144 primer mixes utilizing sequence-specific primers (PCR-SSP). *Tissue Antigens 1995 Nov;46(5):355-67. -67*.

28. Yim, J. J. *et al.* The association between microsatellite polymorphisms in intron II of the human Toll-like receptor 2 gene and tuberculosis among Koreans. *Genes Immun. 2006 Mar* ***7***, 150-155.

29. de Weger, R. A., Tilanus, M. G., Scheidel, K. C., van den Tweel, J. G. & Verdonck, L. F. Monitoring of residual disease and guided donor leucocyte infusion after allogeneic bone marrow transplantation by chimaerism analysis with short tandem repeats. *Br J Haematol. 2000 Sep* **110**, 647-653.

30. Richter, E., Weizenegger, M., Rusch-Gerdes, S. & Niemann, S. Evaluation of genotype MTBC assay for differentiation of clinical Mycobacterium tuberculosis complex isolates. *J Clin Microbiol. 2003 Jun* **41**, 2672-2675.

31. Muzio, M. *et al.* Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. *J Immunol. 2000 Jun* **1;164**, 5998-6004.

32. Hopkins, P. A. & Sriskandan, S. Mammalian Toll-like receptors: to immunity and beyond. *Clin Exp Immunol. 2005 Jun* **140**, 395-407.

33. Drake, W. P. *et al.* Cellular recognition of Mycobacterium ESAT-6 and katG peptides in systemic sarcoidosis. *Infect Immun. 2006 Nov* **6;..**.

34. Ebe, Y. *et al.* Proliferative response of peripheral blood mononuclear cells and levels of antibody to recombinant protein from Propionibacterium acnes DNA expression library in Japanese patients with sarcoidosis. *Sarcoidosis Vasc Diffuse Lung Dis 2000 Oct;17(3):256-65.* -65.

35. Yim, J. J. *et al.* A microsatellite polymorphism in intron 2 of human Toll-like receptor 2 gene: functional implications and racial differences. *FEMS Immunol Med Microbiol. 2004 Mar* **8;40**, 163-169.

36. Yim, J. J. *et al.* A microsatellite polymorphism in intron 2 of human Toll-like receptor 2 gene: functional implications and racial differences. *FEMS Immunol Med Microbiol. 2004 Mar* **8;40**, 163-169.

37. Yim, J. J. *et al.* The association between microsatellite polymorphisms in intron II of the human Toll-like receptor 2 gene and tuberculosis among Koreans. *Genes Immun. 2006 Mar* **7**, 150-155.

38. Underhill, D. M., Ozinsky, A., Smith, K. D. & Aderem, A. Toll-like receptor-2 mediates mycobacteria-induced proinflammatory signaling in macrophages. *Proc Natl Acad Sci U S A. 1999 Dec* **7;96**, 14459-14463.

39. Baughman, R. P. & Iannuzzi, M. Tumour necrosis factor in sarcoidosis and its potential for targeted therapy. *BioDrugs. 2003* **17**, 425-431.

40. Detournay, O., Mazouz, N., Goldman, M. & Toungouz, M. IL-6 produced by type I IFN DC controls IFN-gamma production by regulating the suppressive effect of CD4+ CD25+ regulatory T cells. *Hum Immunol. 2005 May* **66**, 460-468.

41. Kim, D. S. *et al.* The value of interleukin-12 as an activity marker of pulmonary sarcoidosis. *Sarcoidosis Vasc Diffuse Lung Dis. 2000 Oct* **17**, 271-276.

42. Shigehara, K. *et al.* Increased circulating interleukin-12 (IL-12) p40 in pulmonary sarcoidosis. *Clin Exp Immunol. 2003 Apr* **132**, 152-157.

43. Bihl, M. P. *et al.* Progressive pulmonary sarcoidosis–a fibroproliferative process potentially triggered by EGR-1 and IL-6. *Sarcoidosis Vasc Diffuse Lung Dis. 2006 Mar* **23**, 38-50.

44. Takizawa, H. *et al.* Increased IL-6 and IL-8 in bronchoalveolar lavage fluids (BALF) from patients with sarcoidosis: correlation with the clinical parameters. *Clin Exp Immunol. 1997 Jan* **107**, 175-181

4

CD14 genetics in sarcoidosis

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Abstract

Sarcoidosis is an inflammatory disease of unknown aetiology most often affecting the lungs, lymph nodes and skin. Infectious agents have been proposed suggesting a role for pattern-recognition receptors such as Toll-like receptors (TLR) in disease pathogenesis, with a special interest in TLR-2. CD14 interacts with various pathogen-associated molecular patterns and is capable of forming a receptor complex with TLR-2. The aim of this study was to investigate possible genetic differences in the promoter region of CD14 based on the hypothesis that these will predispose for sarcoidosis or will influence its disease course. In the first part of this chapter allele frequencies of CD14 promoter single nucleotide polymorphisms (SNPs) -550C/T and -159C/T in 178 patients and 195 healthy controls are presented which has been published in 2007. No associations were found between CD14 promoter polymorphisms and susceptibility of sarcoidosis (n=102). The T allele frequency of SNP -550C/T was significantly higher in patients with Löfgren's syndrome in the study cohort (n=76) but this could not be replicated in a validation cohort of 42 patients with Löfgren's syndrome. The second part of the chapter presents genetic data regarding four CD14 promoter SNPs (including -159C/T and -550C/T) and an increased number of patients and controls. Genotyping and construction of haplotypes was performed in 447 healthy controls and 533 patients, divided in 425 patients with sarcoidosis and 108 patients with Löfgren's syndrome. The prevalence of a low frequency CD14 promoter haplotype was significantly increased in sarcoidosis patients with a chronic disease course compared to healthy controls. No differences were found between healthy controls and patients with Löfgren's syndrome.

In summary, our study suggests that presence of a low frequency haplotype in the CD14 promoter region increases the risk of developing chronic disease in patients already affected by sarcoidosis. Due to the fact that CD14 can act as a co-receptor for TLR-2, this study supports the growing evidence that aberrant TLR-2 function plays a role in part of the patients with sarcoidosis.

Introduction

Sarcoidosis is a systemic disease of unknown origin, primarily affecting the lungs. The disease is characterized by the formation of non-caseating granulomas and a strong cell-mediated immune response, suggesting intracellular pathogens as causative agents. Both intracellular pathogens *Mycobacterium tuberculosis* and *Propionibacterium acnes* have extensively been studied and could well play a role in the pathogenesis of sarcoidosis[1-9].

Recent years have seen a dramatic increase in our understanding of the role of innate immunity in human host defense. Innate immunity can initiate an inflammatory and anti-microbial response, thereby dictating the ensuing adaptive immune response. For granulomatous disorders, such as Crohn's disease and tuberculosis, it has been demonstrated that defects in innate immune responses play a role in disease pathogenesis [10-12].

An important category of innate immunity receptors are the Toll-like receptors (TLR), a family of related transmembrane or endosomal molecules each recognizing a distinct, but limited, repertoire of microbial encoded molecules. A role for TLR-2, recognizing peptidoglycans, lipopeptides and heat shock proteins, in the pathogenesis of sarcoidosis is suggested by several studies [13-15]. Interestingly, TLR-2 is important in the innate immune response against both *Mycobacterium tuberculosis* as well as *Propionibacterium acnes* [16,17]. TLR-2 senses a variety of microbial components and it has been shown that TLR-1 and TLR-6 are co-receptors for TLR-2 [18]. CD14, first characterized as the classical endotoxin receptor, appears to be involved in the immune response against Gram-positive bacteria as well as mycobacteria [19,20] and can also form a complex with TLR-2 [21]. CD14 is expressed in membrane-bound form (mCD14) on the cell surfaces of monocytes and neutrophils, but it is also present in serum as a soluble protein (sCD14). It has been shown that sCD14 can induce an inflammatory response in non-myeloid cells lacking the membrane bound form of the protein [22]. On the other hand, however, sCD14 has been shown to have beneficial properties in protection against LPS induced endotoxin shock [23].

Two single nucleotide polymorphisms (SNPs) in the promoter region of CD14 (-159 C>T and -550 T>C) have been associated with a higher plasma level of the

soluble form [24,25]. Interestingly, it has been shown that soluble CD14 is increased in the bronchoalveolar lavage fluid of patients with active sarcoidosis compared with inactive sarcoidosis [26]. Furthermore, Gazouli *et al* demonstrated an increased prevalence of the -159 T allele in sarcoidosis suggesting that increased soluble CD14 in sarcoidosis can partly be explained by genetic variation [27]. A recent study of Fridlender *et al.* confirmed this finding [28].
Taken together, based on previous studies and its function as co-receptor for TLR-2, CD14 seems an attractive candidate for genetic analysis in sarcoidosis. Our hypothesis is that genetic differences in the CD14 promoter region will derange innate sensing of pathogens in sarcoidosis by an altered CD14 expression and therefore will influence disease susceptibility or disease outcome.

Materials and Methods

Study subjects

One hundred seventy-eight unrelated and randomly selected Dutch Caucasian patients with sarcoidosis were included in the study cohort. In 102 patients, the diagnosis of sarcoidosis was established when clinical findings were supported by histological evidence, and after the exclusion of other known causes of granulomatosis. 76 patients presented with the classical Löfgren's syndrome of fever, erythema nodosum, bilateral hilar lymphadenopathy and joint symptoms. Verbal and written consent was obtained from all subjects, and authorization was given by the Ethics Committee of the St. Antonius Hospital, Nieuwegein. The control subjects comprised 195 healthy Dutch Caucasian employees of the St. Antonius Hospital in The Netherlands. By completing a questionnaire, relevant background information was provided by these volunteers and included medication, ethnicity and hereditary diseases. As a validation cohort of 254 unrelated and randomly selected Caucasian patients with sarcoidosis from a hospital located in a different geographic region in The Netherlands was used. In this group there were 212 sarcoidosis patients and 42 patients with Löfgren's syndrome. Written consent was obtained from these subjects as well as approval by the Ethics Committee of the University Hospital, Maastricht.

Evaluation of pulmonary disease severity

Chest radiographs at presentation, 2 years and 4 years were collected for each patient and assessed blind by a pulmonary physician for disease severity using standard radiographic staging for sarcoidosis. In brief, this comprises five stages: stage 0 = normal, stage I = bilateral hilar lymphadenopathy (BHL), stage II = BHL and parenchymal infiltration, stage III = parenchymal infiltration without BHL, stage IV = irreversible fibrosis with loss of lung volume. Radiographic evolution over a 4 year period was available for 178 patients and was categorized as follows; A: normalization or improvement towards stage I (n=44), B: persistent stage II/III or progression into that direction (n=35), and C: stable stage IV or progression towards this stage (n=23) [29]. Patients who had been diagnosed with Löfgren's syndrome at presentation (n= 76) were considered as a distinct group with radiographic evolution not exceeding stage I. In the validation cohort there were 42 patients with Löfgren's syndrome, 72 patients were categorized as stage A, 105 patients as stage B and 35 patients as stage C. Patients with Löfgren's syndrome or stage A were defined as acute/self-remitting disease and patients with stages B or C as chronic disease.

Analysis of CD14 gene polymorphisms

Identification of the CD14 –159C>T polymorphism was performed using the dual labeled allele specific oligonucleotides 5'-[FAM]- CTGTTACGGCCCCCCT-[NFQ] and 5'-[VIC™] CCTGTTACGGTCCCCCT-[NFQ] together with forward primer 5'-CCCTTCCTTTCCTGGAAATATTGCA- and reverse primer 5'-CTAGA TGCCCTGCAGAATCCTT- following manufacturers' instructions (Custom TaqMan® SNP Genotyping Assay, Applied Biosystems). Identification of the CD14 –550C>T polymorphism was performed using allele specific oligonucleotides 5'-[FAM]-AAAAGGAAGTTGGTCTAAA-[NFQ] and 5'-[VIC™] AAGAAAAGGAAGTT AGTCTAAA-[NFQ] together with forward primer 5'-AACCTCATCTGTTGG CACCAA- and reverse primer 5'-AAGAAAGTTATTACTTAATCAAAGGAGCAAGGA.

Statistical analysis

Allele and genotype frequencies were calculated for each locus and tested for Hardy-Weinberg equilibrium in controls. Association studies were analysed by chi-square testing using 2x3 and 2x2 contingency tables for genotype and allele frequencies, respectively. P-values were corrected using a Bonferroni correction for multiple testing.

Results

Distribution of CD14 polymorphisms in patients and healthy controls

Table 1 summarizes the allele and genotype frequencies of the investigated CD14 SNPs in healthy controls, the study cohort and the validation cohort. The SNPs did not deviate from Hardy-Weinberg equilibrium in controls. In the study cohort, there was a non- significant reduction of the -159 T allele frequency in patients with Löfgren's syndrome compared to healthy controls (p=0.06, corrected for multiple testing p=0.12). Furthermore, a significant reduction of -550 T allele frequency was found in patients with Löfgren's syndrome compared to healthy controls (p=0.002, corrected for multiple testing p=0.004). No differences were found between sarcoidosis patients and controls. In our validation cohort, however, no differences were found in allele frequencies of both SNPs between controls and sarcoidosis patients or patients with Löfgren's syndrome. In both cohorts no differences were found when comparing different radiographic phenotypes of sarcoidosis patients.

			Study cohort		Validation cohort				Study cohort		Validation cohort	
SNP	**Allele**	**Ctr**	**Sar**	**Löf**	**Sar**	**Löf**	**Genotype**	**Ctr**	**Sar**	**Löf**	**Sar**	**Löf**
-159 C/T	C	0.51	0.53	0.59	0.50	0.50	CC	56 (0.29)	29 (0.28)	32 (0.42)	50 (0.24)	12 (0.29)
	T	0.49	0.47	0.41	0.50	0.50	CT	87 (0.45)	50 (0.49)	28 (0.39)	112 (0.53)	18 (0.43)
							TT	52 (0.27)	23 (0.23)	16 (0.21)	50 (0.24)	12 (0.29)
-550 C/T	C	0.79	0.74	0.66	0.77	0.79	CC	122 (0.63)	54 (0.53)	32 (0.42)	128 (0.60)	26 (0.62)
	T	0.21	0.26	0.34	0.23	0.21	CT	65 (0.33)	43 (0.42)	36 (0.47)	70 (0.33)	14 (0.33)
							TT	8 (0.04)	5 (0.04)	8 (0.10)	14 (0.07)	2 (0.05)

Table 1. Allele and genotype frequencies of CD14 polymorphisms in patients and controls. -159 C/T = rs2569190, -550C/T =rRs5744455 Ctr = healthy controls, Sar = sarcoidosis patients, Löf= patients with Löfgren's syndrome. Genotype data are shown as total number of alleles with allele frequencies in parentheses. Cumulative frequencies do not add up to 1.0 due to rounding.

Interim discussion

Previous studies regarding the CD14 -159 C>T single nucleotide polymorphism demonstrated a higher prevalence of the T allele in sarcoidosis patients [27,28]. In the present study we did not find a difference in T allelic carriage of this polymorphism in sarcoidosis. Regarding the study published by Gazouli *et al* [27], in both the study cohort as well as the validation cohort of Dutch sarcoidosis patients the T allele carriage at CD14-159 was comparable with Greek sarcoidosis patients. Interestingly, there was a significant difference in -159 T allelic carriage between the Dutch and Greek healthy controls ($p<0.0001$, data not shown). In other words, comparable T allele carriage in patients with the same disease can lead to different conclusions based on their matched controls, making interpretation difficult. The allelic distribution in Dutch healthy controls is in congruence with the HapMap-CEU cohort found in the dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP), indicating normal prevalences of the -159 minor and major allele in Dutch controls subjects. Regarding the recent published study by Fridlender *et al.* [28], no allelic differences between both control groups were found. Their cohort of sarcoidosis patients was, similar to our cohort, relatively small, 74 vs. 102 patients respectively. Their study would have been more robust if the results were replicated in a validation cohort as performed in the present study. Overall, the published data so far cannot provide a strong link between the CD14 -159 C>T polymorphism and susceptibility of sarcoidosis.

In our study cohort we found a significantly increased prevalence of the T allele at CD14 –550 and a non-significant decrease in T allele frequency at CD14 –159 in patients with Löfgren's syndrome compared to healthy controls. One could speculate that some patients with Löfgren's syndrome, based on this genetic variation, have a higher amount of sCD14 present in serum or BAL fluid, as was already demonstrated in patients with active sarcoidosis [26]. If this predisposes for the development of Löfgren's syndrome or influences disease course remains to be seen. These genetic associations, however, could not be confirmed in an independent cohort of Löfgren patients.

Overall, our data do not support the previous associations found between the CD14 -159 C>T polymorphism and susceptibility of sarcoidosis. Furthermore, an association between CD14 promoter polymorphism -550 C>T and Löfgren's syndrome was found but could not be replicated in an independent cohort.

Addendum

In 2007 we published the above data on genetic variation in two CD14 promoter polymorphisms in Dutch sarcoidosis and Löfgren patients [30]. An association between the two tested SNPs and Löfgren's syndrome was suggested, but could not be replicated in a validation cohort. In our original cohort there were 76 patients with Löfgren's syndrome compared to only 42 in the validation cohort. In theory, it could be that the differences found were not replicated in the validation cohort due to inadequate statistical power although the found allelic frequencies in this second Löfgren population were almost identical compared to the healthy controls (table 1). To enhance understanding of the role of CD14 polymorphisms in sarcoidosis we enlarged our study population and SNP selection. We recently genotyped 4 SNPs (including -159 C>T and -550 C>T) in the promoter region of CD14 in 533 patients and 447 healthy controls using a custom Illumina goldengate bead SNP assay, performed in accordance with the manufacturer's recommendations (Illumina Inc, San Diego, USA).The patient group comprised of 425 sarcoidosis and 108 Löfgren patients. Ethical considerations, informed consent and evaluation of pulmonary disease severity were performed as described in the Materials and Methods section. Of the 178 patients in the study cohort initially described in this chapter who were genotyped with dual labeled allele specific oligonucleotides (Custom TaqMan® SNP Genotyping Assay), 127 patients were also tested using the bead SNP assay. Comparison of both genotyping techniques revealed that -159 C>T genotypes and -550 C>T genotypes were 100% and 97% identical, respectively (data not shown). Data from an additional cohort of 54 Löfgren patients was now available for comparison with the published data and is shown in table 2.

SNP	Allele	Control	Löfgren 2007st.	Löfgren 2007 val.	Löfgren 2010	Löfgren Total	p-value
-159 C/T	C	0.51	0.59	0.50	0.53	0.55	0.30
	T	0.49	0.41	0.50	0.47	0.45	
-550 C/T	C	0.79	0.66	0.79	0.81	0.74	0.33
	T	0.21	0.34	0.21	0.19	0.26	

Table 2. Allele frequencies of CD14 polymorphisms in 3 cohorts of patients with Löfgren's syndrome and healthy controls. A total of 447 controls and 172 patients with Löfgren's syndrome were genotyped. Löfgren 2007 st.= study cohort (n=76). Löfgren 2007 val.=validation cohort (n=42). Löfgren 2010 = the additional cohort of Löfgren patients (n=54).

The data in table 2 now clearly demonstrate the absence of a genetic association between CD14 -159 C>T and -550 C>T SNP and Löfgren' syndrome. The data of all four CD14 promoter polymorphisms tested using the SNP bead assay are given in table 3. No association between rs2569192 or rs5477771 and sarcoidosis or patients with Löfgren' syndrome was found. Linkage was found between rs5744441 and rs5744455 (-550C/T), depicted in figure 1.

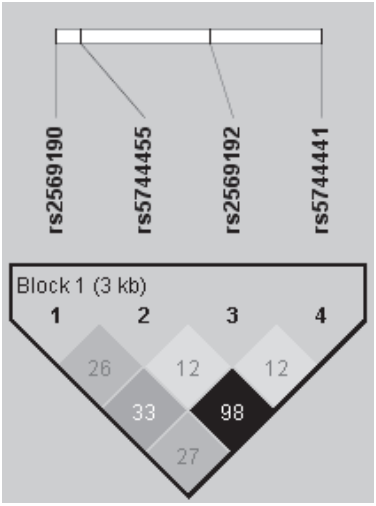

Figure 1. Pair-wise linkage disequilibrium values of CD14 single-nucleotide polymorphisms in a Dutch population. Values of the pair-wise r^2 (x100) are shown in blocks.

SNP	Allele	Control	Sarcoidosis	Löfgren	Genotype	Control	Sarcoidosis	Lofgren	P value [$P_{corrected}$] Ctr vs. Pts	Ctr vs. Sar	Ctr vs. Löf
rs2569190	T	0.47	0.46	0.41	TT	0.24	0.22	0.17	0.37 [ns]	0.75 [ns]	0.09 [ns]
(-159 C/T)	C	0.53	0.54	0.59	TC	0.47	0.48	0.47			
					CC	0.29	0.29	0.36			
rs5744455	T	0.24	0.23	0.28	TT	0.06	0.06	0.07	0.86 [ns]	0.79 [ns]	0.18 [ns]
(-550C/T)	C	0.76	0.77	0.72	TC	0.34	0.34	0.42			
					CC	0.59	0.60	0.51			
rs2569192	G	0.73	0.72	0.72	GG	0.52	0.51	0.50	0.75 [ns]	0.74 [ns]	1 [ns]
	C	0.27	0.28	0.28	GC	0.41	0.41	0.44			
					CC	0.07	0.08	0.06			
rs5744441	T	0.24	0.23	0.30	TT	0.06	0.06	0.07	0.74 [ns]	0.79 [ns]	0.07 [ns]
	C	0.76	0.77	0.70	TC	0.35	0.35	0.45			
					CC	0.59	0.60	0.47			

Table 3. Allele and genotype frequencies of CD14 polymorphisms in patients and controls. Sarcoidosis means patients without Löfgren's syndrome. Ctr. = controls. Pts = all patients. Sar = sarcoidosis patients. Löf = patients with Löfgren's syndrome. P values are based on allele frequency differences. P values corrected for multiple testing (Bonferroni correction) are shown in brackets. ns = non significant. At location rs2569190 14 controls, 33 sarcoidosis patients and 2 patients with Löfgren's syndrome had a missing genotype. At location rs256912 2 patients with Löfgren's syndrome had a missing genotype. Not all frequencies add up to 1.0 due to rounding.

Haplotype frequencies were subsequently determined using Haploview version 4.0 [31]. The construction of haplotypes based on 4 SNPs produced a total of 4 different haplotypes. Haplotype frequencies are summarized in table 4. The frequency of haplotype 4 was significantly increased in patients with sarcoidosis compared to healthy controls. Separate analysis of patients with a self-remitting or a chronic disease course revealed that the significant difference depends on a higher frequency of haplotype 4 in patients with chronic disease. No significant differences were found when comparing haplotype frequencies between healthy controls and Löfgren patients. However, the data suggest a lower frequency of haplotype 1 in patients with Löfgren's syndrome.

Haplo type	SNP position				Controls	Sarcoidosis	Löfgren	Sarcoidosis		P value		
								A	BC	Ctr vs S	Ctr vs L	Ctr vs BC
	rs2569190	rs5744455	rs2569192	rs5744441								
1	C	T	C	T	0.47 (410)	0.46 (36)	0.40 (84)	0.45 (134)	0.44 (105)	0.71	0.07	0.44
2	T	T	G	T	0.28 (241)	0.28 (223)	0.28 (59)	0.29 (86)	0.29 (69)	0.78	0.88	0.67
3	T	C	C	G	0.24 (204)	0.23(177)	0.29 (60)	0.24 (72)	0.22 (53)	0.64	0.11	0.72
4	T	T	C	T	0.01 (9)	0.02 (18)	0.01 (3)	0.02 (5)	0.04 (9)	**0.04**	0.62	**0.003**

Table 4. CD14 haplotype frequencies between controls, sarcoidosis (including self-remitting vs. chronic disease) and Löfgren patients. Frequencies are calculated from the number of alleles. Absolute numbers are in parentheses. Clinical phenotypes are based on radiographic evolution over a minimum 4 year period. A = normalization of improvement towards stage I (n=154), B = persistent stage II/III or progression in that direction (n=61) and C = stable stage IV or progression towards this stage (n=61). Ctr = healthy controls, S = sarcoidosis patients without Löfgren's syndrome, L = patients with Löfgren's syndrome, BC = sarcoidosis patients with chronic course (with and without fibrosis), n= 122. P values were calculated using 2x2 contingency tables. Significant values are printed in bold. Not all frequencies add up to 1.0 due to rounding.

Final discussion

In this study we investigated 4 different single nucleotide polymorphisms located in the promoter region of the gene encoding CD14. We found a significant increased prevalence of a low frequency CD14 promoter haplotype in patients with a chronic course of sarcoidosis. An association between genetic variation in the CD14 promoter was already suggested in previous studies [27,28]. The fact that the frequency of this haplotype in healthy controls is only 1% and in patients with chronic sarcoidosis 4% indicates that genetic variation in the CD14 promoter region is not a major disease susceptibility locus in sarcoidosis. However, it is important to state that sarcoidosis might not be a single disease but rather a collection of different granulomatous disorders, each with its own inducing antigen. CD14 can act as a co-receptor for TLR-2 and appears to be involved in the immune response against Gram-positive bacteria as well as mycobacteria [19,20]. There is increasing evidence that TLR-2 plays a role in sarcoidosis disease pathogenesis [13-15], however, at present it remains unknown in which distinct group of patients the TLR-2 pathway could be important. Interestingly, the -159C/T polymorphism has been associated with an increased risk of developing tuberculosis [20]. Despite evidence demonstrating the higher promoter activity of the -159TT genotype, the effect on sCD14 expression levels *in vivo* remains controversial [24,32]. It could be that it is not -159C/T that influences CD14 expression, but an adjacent polymorphism in the promoter region present on haplotye 4 found in our study. In light of a possible role for *Mycobacterium tuberculosis* in sarcoidosis it is interesting that a higher level of sCD14 can inhibit internalization of *M. tuberculosis* by competitive inhibition between *M. tuberculosis* and mCD14 [33,34]. If *M. tuberculosis* is a cause of sarcoidosis, the presence of haplotype 4 in patients already affected by sarcoidosis could result in a higher risk of developing chronic disease based on a different interaction between *M. tuberculosis* and the innate immune system. Apart from *M. tuberculosis*, CD14 seems also to be involved in the size of granulomas induced by intraperitoneal administration of *Propnionibacterium acnes* in mice [35]. In order to fully appreciate the impact of our genetic data, further experiments are needed in order to test whether haplotype 4 does influence CD14 expression and interaction with TLR-2 agonists such as *M. tuberculosis* or *P. acnes*.

No significant differences in CD14 genotype or haplotype frequencies were found in patients with Löfgren's syndrome compared to healthy controls. A non-significant decrease in the most common CD14 promoter haplotype was found. Functional experiments addressing possible differences in CD14 function between the 4 haplotypes found are needed to indicate clinical relevance of this finding in patients with Löfgren's syndrome.

In summary, our study suggests that presence of a low frequency haplotype in the CD14 promoter region increases the risk of developing chronic disease in patients already affected by sarcoidosis. Due to the fact that CD14 can act as a co-receptor for TLR-2, this study supports the growing evidence that aberrant TLR-2 function plays a role in a subset of patients with sarcoidosis.

Reference List

1. Abe, C., Iwai, K., Mikami, R. & Hosoda, Y. Frequent isolation of Propionibacterium acnes from sarcoidosis lymph nodes. *Zentralbl. Bakteriol. Mikrobiol. Hyg. A* **256**, 541-547 (1984).
2. Dubaniewicz, A., Kampfer, S. & Singh, M. Serum anti-mycobacterial heat shock proteins antibodies in sarcoidosis and tuberculosis. *Tuberculosis. (Edinb.)* **86**, 60-67 (2006).
3. Eishi, Y. *et al.* Quantitative analysis of mycobacterial and propionibacterial DNA in lymph nodes of Japanese and European patients with sarcoidosis. *J. Clin. Microbiol.* **40**, 198-204 (2002).
4. Fite, E., Fernandez-Figueras, M. T., Prats, R., Vaquero, M. & Morera, J. High prevalence of Mycobacterium tuberculosis DNA in biopsies from sarcoidosis patients from Catalonia, Spain. *Respiration* **73**, 20-26 (2006).
5. Gazouli, M. *et al.* Assessment of mycobacterial, propionibacterial, and human herpesvirus 8 DNA in tissues of greek patients with sarcoidosis. *J. Clin. Microbiol.* **40**, 3060-3063 (2002).
6. Gupta, D., Agarwal, R., Aggarwal, A. N. & Jindal, S. K. Molecular evidence for the role of mycobacteria in sarcoidosis: a meta-analysis. *Eur. Respir. J.* **30**, 508-516 (2007).
7. Iio, K. *et al.* Experimental pulmonary granuloma mimicking sarcoidosis induced by Propionibacterium acnes in mice. *Acta Med. Okayama* **64**, 75-83 (2010).
8. McCaskill, J. G. *et al.* Pulmonary immune responses to Propionibacterium acnes in C57BL/6 and BALB/c mice. *Am. J. Respir. Cell Mol. Biol.* **35**, 347-356 (2006).
9. Song, Z. *et al.* Mycobacterial catalase-peroxidase is a tissue antigen and target of the adaptive immune response in systemic sarcoidosis. *J. Exp. Med.* **201**, 755-767 (2005).
10. Marks, D. J. *et al.* Defective acute inflammation in Crohn's disease: a clinical investigation. *Lancet* **367**, 668-678 (2006).
11. Rosas-Taraco, A. G. *et al.* CD14 C(-159)T polymorphism is a risk factor for development of pulmonary tuberculosis. *J. Infect. Dis.* **196**, 1698-1706 (2007).
12. Yim, J. J. *et al.* The association between microsatellite polymorphisms in intron II of the human Toll-like receptor 2 gene and tuberculosis among Koreans. *Genes Immun.* **7**, 150-155 (2006).
13. Chen, E. S. *et al.* Serum amyloid A regulates granulomatous inflammation in sarcoidosis through Toll-like receptor-2. *Am. J. Respir. Crit Care Med.* **181**, 360-373 (2010).
14. Veltkamp, M. *et al.* Linkage between Toll-like receptor (TLR) 2 promotor and intron polymorphisms: functional effects and relevance to sarcoidosis. *Clin. Exp. Immunol.* **149**, 453-462 (2007).
15. Wiken, M., Grunewald, J., Eklund, A. & Wahlstrom, J. Higher monocyte expression of TLR2 and TLR4, and enhanced pro-inflammatory synergy of TLR2 with NOD2 stimulation in sarcoidosis. *J. Clin. Immunol.* **29**, 78-89 (2009).

16. Bowdish, D. M. *et al.* MARCO, TLR2, and CD14 are required for macrophage cytokine responses to mycobacterial trehalose dimycolate and Mycobacterium tuberculosis. *PLoS. Pathog.* **5**, e1000474 (2009).

17. Jugeau, S. *et al.* Induction of toll-like receptors by Propionibacterium acnes. *Br. J. Dermatol.* **153**, 1105-1113 (2005).

18. Kirschning, C. J. & Schumann, R. R. TLR2: cellular sensor for microbial and endogenous molecular patterns. *Curr. Top. Microbiol. Immunol.* **270**, 121-144 (2002).

19. Dziarski, R., Ulmer, A. J. & Gupta, D. Interactions of CD14 with components of gram-positive bacteria. *Chem. Immunol.* **74**, 83-107 (2000).

20. Kang, Y. A. *et al.* Association between the -159C/T CD14 gene polymorphism and tuberculosis in a Korean population. *FEMS Immunol. Med. Microbiol.* **57**, 229-235 (2009).

21. Takeuchi, O. *et al.* Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity.* **11**, 443-451 (1999).

22. Tapping, R. I. & Tobias, P. S. Soluble CD14-mediated cellular responses to lipopolysaccharide. *Chem. Immunol.* **74**, 108-121 (2000).

23. Jacque, B. *et al.* Mice expressing high levels of soluble CD14 retain LPS in the circulation and are resistant to LPS-induced lethality. *Eur. J. Immunol.* **36**, 3007-3016 (2006).

24. Baldini, M. *et al.* A Polymorphism* in the 5' flanking region of the CD14 gene is associated with circulating soluble CD14 levels and with total serum immunoglobulin E. *Am. J. Respir. Cell Mol. Biol.* **20**, 976-983 (1999).

25. Inoue, Y. *et al.* CD14 -550 C/T, which is related to the serum level of soluble CD14, is associated with the development of respiratory syncytial virus bronchiolitis in the Japanese population. *J. Infect. Dis* **195**, 1618-1624 (2007).

26. Striz, I. *et al.* Soluble CD14 is increased in bronchoalveolar lavage of active sarcoidosis and correlates with alveolar macrophage membrane-bound CD14. *Am. J. Respir. Crit Care Med.* **151**, 544-547 (1995).

27. Gazouli, M. *et al.* CARD15/NOD2, CD14, and toll-like receptor 4 gene polymorphisms in Greek patients with sarcoidosis. *Sarcoidosis. Vasc. Diffuse. Lung Dis.* **23**, 23-29 (2006).

28. Fridlender, Z. G. *et al.* Association between CD14 gene polymorphisms and disease phenotype in sarcoidosis. *Respir. Med* **104**, 1336-1343 (2010).

29. Grutters, J. C. *et al.* Analysis of IL6 and IL1A gene polymorphisms in UK and Dutch patients with sarcoidosis. *Sarcoidosis Vasc Diffuse Lung Dis 2003 Mar;20(1):20-7. -7*.

30. Veltkamp, M. *et al.* CD14 genetics in sarcoidosis patients; who's in control? *Sarcoidosis. Vasc. Diffuse. Lung Dis.* **24**, 154-155 (2007).

31. Barrett, J. C., Fry, B., Maller, J. & Daly, M. J. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics.* **21**, 263-265 (2005).

32. Heesen, M. *et al.* Lack of association between the -260 C–>T promoter polymorphism of the endotoxin receptor CD14 gene and the CD14 density of unstimulated human monocytes and soluble CD14 plasma levels. *Intensive Care Med* **27**, 1770-1775 (2001).

33. Lewthwaite, J. C. *et al.* Mycobacterium tuberculosis chaperonin 60.1 is a more potent cytokine stimulator than chaperonin 60.2 (Hsp 65) and contains a CD14-binding domain. *Infect. Immun.* **69**, 7349-7355 (2001).

34. Peterson, P. K. *et al.* CD14 receptor-mediated uptake of nonopsonized Mycobacterium tuberculosis by human microglia. *Infect. Immun.* **63**, 1598-1602 (1995).

35. Uchida, T. *et al.* Involvement of CD14 in lipopolysaccharide- induced liver injury in mice pretreated with Propionibacterium acnes. *Pathobiology* **71**, 246-252 (2004).

5

Toll-like receptor (TLR) 9 genetics and function in sarcoidosis

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Abstract

Sarcoidosis is a systemic disorder characterized by the formation of non-caseating granulomas in variable organs. Toll-like receptor (TLR) 9 is important in the innate immune response against both *Mycobacterium tuberculosis* and *Propionibacterium acnes*, candidate causative agents in sarcoidosis. The aim of our study was to investigate possible genetic and functional differences in TLR-9 between patients and controls. TLR-9 single nucleotide polymorphisms were genotyped in 533 patients, divided into a study cohort and validation cohort, and 185 healthy controls. Furthermore, part of the promoter as well as the entire coding region of the TLR-9 gene were sequenced in 20 patients in order to detect new mutations. No genetic differences were found between patients and controls. In order to test TLR-9 function, peripheral blood mononuclear cells (PBMCs) of 12 healthy controls and 12 sarcoidosis patients were stimulated with a TLR-9 agonist and the induction of interleukin 6 (IL-6), interferon-gamma (IFN-γ) and interleukin 23 (IL-23) was measured. Sarcoidosis patients produce significantly less IFN-γ upon stimulation with different stimuli. Regarding IL-23 production, a significant difference between patients and controls only was found after stimulation with the TLR-9 agonist. In conclusion, we did not find genetic differences in the TLR-9 gene between sarcoidosis patients and controls. Sarcoidosis patients produce less IFN-γ regardless of the stimulating agent, probably reflecting the anergic state often seen in their peripheral blood T-lymphocytes. The differences in TLR-9 induced IL-23 production could indicate that functional defects in the TLR-9 pathway of sarcoidosis patients play a role in disease susceptibility or evolution.

Introduction

Sarcoidosis is a systemic disorder of unknown aetiology leading to the formation of non-caseating granulomas in variable organs such as lungs, lymph nodes and skin. The disease is characterized by a strong cell-mediated immune reaction, making microbial pathogens such as viruses or intracellular bacteria leading candidates as causative agents. Especially the intracellular bacteria *Mycobacterium tuberculosis* and *Propionibacterium acnes* have been extensively studied and may well play a role in disease pathogenesis [1-5].

In recent years, many genetic association studies have been performed with an emphasis on innate immunity [6-12]. An important category of innate immunity receptors are the Toll-like receptors (TLR), a family of related transmembrane or endosomal molecules each recognizing a distinct, but limited, repertoire of microbial encoded molecules. Two of the TLR genes, TLR-4 and TLR-9, are located in close vicinity of chromosomal positions that have shown linkage in a previous study on predisposing gene loci in sarcoidosis [13]. TLR-4 is an essential receptor for the recognition of Lipopolysaccharide (LPS), unique to the cell wall of gram-negative bacteria. Further studies on a genetic role of TLR-4 in sarcoidosis provide conflicting results. In a recent study all 10 known TLR genes on 7 different chromosomal loci were tested for linkage with sarcoidosis [8]. Once again, a significant linkage between a locus on chromosome 9 (near TLR-4) and sarcoidosis was found. These results could partly explain the association found by Pabst and colleagues between a functional TLR-4 polymorphism and a chronic course of sarcoidosis [7]. However, in subsequent case-control analysis, no association was found with this functional TLR-4 polymorphism Asp299Gly and sarcoidosis, a result in concordance with other studies showing no genetic association [6,10].

The endosomal localized receptor TLR-9, capable of recognizing unmethylated nucleic acid motifs, is one of the most important receptors in the initiation of protective immunity against intracellular pathogens and necessary for an adequate immune response against both *M. tuberculosis* and *P. acnes* [14,15]. TLR-9 is primarily expressed in B cells, plasmacytoid dendritic cells (pDC) and monocytes/macrophages[16]. Regarding the genetic role of TLR-9 in sarcoidosis, both linkage

studies also reach conflicting results. In the most recent study by Schurmann and colleagues [8], a sub analysis regarding sib-pair families (families with two or more siblings with sarcoidosis) showed significant transmission distortion for a marker located near the TLR-9 gene on chromosome 3p. A significant linkage of a TLR-9 locus with sarcoidosis, however, was not found. In our opinion, the above results do not rule out a genetic role for TLR-9 in the pathogenesis of sarcoidosis and warrants further study.

Taken together, when considering a causative role for intracellular pathogens in the pathogenesis of sarcoidosis, the innate immunity receptor TLR-9, based on its involvement in the immune response against intracellular pathogens and its genomic location, seems an attractive candidate for genetic and functional analysis in sarcoidosis research. We hypothesize that alterations in TLR-9 function are involved in the aberrant immune response characterizing sarcoidosis and therefore are more prevalent in sarcoidosis patients. To address this hypothesis, we performed both a genetic and functional analysis of TLR-9 in sarcoidosis patients and healthy controls.

Materials and Methods

Study subjects for genetic analysis

150 unrelated and randomly selected Dutch Caucasian patients presenting with sarcoidosis at the St. Antonius Hospital (84 men and 66 women) were included in the study cohort. In 102 patients, the diagnosis of sarcoidosis was established when clinical findings were supported by histological evidence, and after the exclusion of other known causes of granulomatosis. 48 patients presented with the classical Löfgren's syndrome of fever, erythema nodosum, bilateral hilar lymphadenopathy and joint symptoms. Verbal and written consent was obtained from all subjects, and authorization was given by the Ethics Committee of the St. Antonius Hospital, Nieuwegein. The control subjects comprised 185 healthy Dutch Caucasian employees of the St Antonius Hospital in the Netherlands. By completing a questionnaire, relevant background information was provided by these volunteers and included medication, ethnicity and hereditary diseases. As a validation cohort,

383 unrelated and randomly selected Caucasian patients with sarcoidosis (190 men and 193 women, 323 patients with non-Lofgren sarcoidosis and 60 patients with Löfgren's syndrome). All patients were diagnosed as described above and written consent was obtained from these subjects as well as approval by the Ethics Committee of the St. Antonius Hospital, Nieuwegein

Evaluation of pulmonary disease severity

Pulmonary disease severity of sarcoidosis patients at presentation was evaluated by chest radiography and pulmonary function testing. Longitudinal chest radiographs for each patient were examined and compared to determine disease outcome. Chest radiographs at presentation, 2 years and 4 years were collected for each patient and assessed blind by a pulmonary physician for disease severity using standard radiographic staging for sarcoidosis. In brief, this comprises five stages: stage 0: normal, stage I: bilateral hilar lymphadenopathy (BHL), stage II: BHL and parenchymal infiltration, stage III: parenchymal infiltration without BHL, stage IV: irreversible fibrosis with loss of lung volume. Radiographic evolution over a minimum 4 year period was available for 313 patients and was categorized as follows: A (normalization of improvement towards stage I), B (persistent stage II/III or progression in that direction, and C (stable stage IV or progression towards this stage) [17]. Patients who have been diagnosed with Löfgren's syndrome at presentation were considered as a distinct group with radiographic evolution not exceeding stage I.

Analysis of genetic polymorphisms in the TLR-9 gene

Genomic DNA extracted from all subjects in the study cohort and controls was genotyped for 4 different TLR-9 single nucleotide polymorphisms using sequence-specific primers (SSPs) and polymerase chain reaction or Taqman genotyping assay when appropriate (-1486). Selection of the polymorphisms was based on a minor allele frequency of more than 10% [18] and functionality [19]. The polymorphisms were located at the following nucleotide positions: -1486 (rs187084, promoter region), -1237 (rs5743839, promoter region), +1173 (rs352139, intron1/exon2) and +2848 (rs352140, exon2). Identification of the polymorphism at location –1486

was performed using the dual labeled allele specific oligonucleotides 5′-[FAM]-AATGACACGGACCCGT-[NFQ] and 5′-[VIC™]-TCAGCTTCTTAA GGGCA-[NFQ] together with forward primer 5′-CGTCTTATTCCCCTGCTGGAA and reverse primer 5′-TGGGCACTGTACTGGATCCT following manufacturer's instructions (Custom TaqMan® SNP Genotyping Assay, Applied Biosystems). For the identification of the polymorphism at position -1237 the sequence-specific forward primers 5′-CATATGAGACTTGGGGGAGTTTT and 5′-ATATGAGACTTGGGGGAGTTTC were combined with the reverse primer 5′-ACTAGGTCCCTCCTC TGCT leading to an expected PCR product size of 231 bp. For identification of the polymorphism at position +1173 the sequence-specific forward primers 5′-AAGT GGAGTGGGTGGAGGTA and 5′-AAGTGGAGTGGGTGGAGGTG were combined with the reverse primer 5′- ATGCGGTTGGAGGACAAGGA leading to an expected PCR product size of 284 bp. For identification of the polymorphism at position +2848 the sequence-specific forward primers 5′- ACTCATTCACG GAGCTACCG and 5′-CACTCATTCACGGAGCTACCA were combined with the reverse primer 5′- TGGAAGAAGTGCAGATAGAGGT leading to an expected PCR product size of 259 bp. In all primer mixes we included control primers 5′-ATGATGTTGACCTTTCCAGGG and 5′-GCAACTGATGAAAAGTTACAGAA leading to an expected PCR product size of 720bp. All PCR reactions were run under identical conditions as previously described[20]. In the validation cohort, one of the two promoter polymorphisms was studied (-1486, (rs187084) as well as the exon 2 polymorphism (+2848, rs352140). Analysis was performed using a custom Illumina goldengate bead SNP assay following the manufacturer's recommendations.

Sequencing of the promoter and exons of TLR-9

In 20 patients (including 7 patients with Löfgren's syndrome) the last 300bp of the promoter in combination with the entire coding region (2 exons) of TLR-9 was sequenced (figure 1). The following primers were used; 5′-TCTAGGGGCTGAATGTGACC with 5′- ACAACCCGTCACTGTTGCTT (promoter/exon1), 5′- GGAGAGAGGGGTTGGAAGAT with 5′-GCATTCAGCCAGGAGAGAGA (exon2), 5′- CTGCGTGTGCTCGATGTG with 5′-AGCCACGAAGCTGAAGTTGT (exon2), 5′- GGTGCTAGACCTGTCCCACA with

5'- CACAGGTGGAAGCAGTACCA (exon2) and 5'- TCAGCATCTTTGCACAGGAC with 5'- CCTCTGTCCCGTCTTTATGG (exon2). The PCR mix consisted of 2.5µl PCR buffer, 1.5µl $MgCl_2$ (25mM), 0.5µl of each dNTP (1.25mM), 0,25µl HotStarTaq (all Applied Biosystems) and 16.25µl demineralised water. Furthermore, 120ng of DNA together with 5 pmol of each primer was added in order to obtain a reaction volume of 25µl. Cycling parameters were 10 min at 95°C followed by 30 cycles of 40 sec at 94°C, 1min at 59°C and 90 sec at 72°C and finally 3 min at 72°C followed by 4 min at 4°C. The PCR products were purified using the Qiaquick PCR purification kit (Qiagen) following manufacturer's instructions. Subsequently, sequencing was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) following the manufacturer's instructions on a ABI PRISM® 3100-*Avant* Genetic Analyzer (Applied Biosystems). The results were analysed with ABI PRISM® Seqscape® Software Version 2.0 (Applied Biosystems)

Analysis of TLR-9 function

Blood samples were collected from 12 randomly selected Dutch sarcoidosis patients visiting our outpatient clinic and 12 healthy Dutch employees from our hospital (all included in the cohort, see Study subjects for genetic analysis). Six patients used immunosuppressive therapy such as prednisolon or methotrexate. Peripheral blood mononuclear cells (PBMC) were isolated from venous blood using Ficoll-Paque centrifugation and seeded on 24-well plates at a density of 400.000 cells per well. PBMCs were stimulated with the TLR-9 agonist ODN2216 (InvivoGen) in combination with Phytohaemagglutinin (PHA), 42µg, Murex). PHA alone (42µg) and the control oligonucleotide for the TLR-9 ligand (ODN2216Control, InvivoGen) in combination with PHA (42µg) were used as positive and negative controls, respectively. PHA was used in addition to the TLR-9 agonist and TLR-9 control oligonucleotide due to the fact that stimulation with oligo-nucleotides only did not induce measurable amounts of cytokines. All stimulations were performed in a final volume of 1200µl RPMI 1640 (Gibco) containing 10% heat-inactivated FCS (Gibco) and 1% Clindamycin/Streptomycin. After a 24-hrs incubation period at 37°C in humidified air containing 5% CO_2, supernatant was collected. IL-6 was chosen as a pro-inflammatory cytokine in order to rule out the possibility that

sarcoidosis patients have a general unresponsiveness for stimuli. Measurement of IFN-γ was chosen as a prototype Th-1 cytokine, IL-23 as a Th-17 cytokine. IL-6, IFN-γ and IL-23 were determined by multiplex immunoassay as described previously [21,22]. To that end, Luminex beads were coated with appropriate catching antibodies (IL-6: clone MQ2-13A5, IL-23: clone eBio473P1, IFN-γ: clone NIB42 (Ready-Set-Go human ELISA kit, eBioscience) and matching biotin conjugated antibodies were used for detection of bound cytokines (IL-6: clone MQ2-39C3, IL-23; clone C8.6, IFN-γ: clone 4S. B3 (Ready-Set-Go human ELISA kit, eBioscience).

Statistical analysis

Allele and genotype frequencies were calculated for each locus and tested for Hardy-Weinberg equilibrium in controls. Association studies were analysed by chi-square testing using 2x3 and 2x2 contingency tables for genotype and allele frequencies, respectively. Data analysis was carried out using the Student's *t*-test. A p value <0.05 was considered significant.

Results

Allelic distribution of TLR-9 polymorphisms

The genotype distribution of the investigated TLR-9 polymorphisms in Dutch sarcoidosis patients and controls are presented in table 1. Genotype data from all populations conformed to Hardy-Weinberg equilibrium. No differences were found between patients and controls regarding the allelic distribution of any of the four polymorphisms. The 2 polymorphisms tested in a validation cohort, one promoter polymorphism and an exon 2 polymorphism, confirmed these data. Also, when comparing different clinical entities within the group of patients, no differences were found.

Sequence analysis of TLR-9

The sequence of the last 300bp of the promoter region, together with the sequence of both exon 1 and exon 2 was determined in 20 patients in order to rule out the presence of new mutations in the gene encoding for TLR-9 (figure 1). In both patients with Löfgren's syndrome (n=7) and patients with non-Löfgren sarcoidosis (n=13) no mutations were found other than the known single nucleotide polymorphisms in the TLR-9 gene in this area.

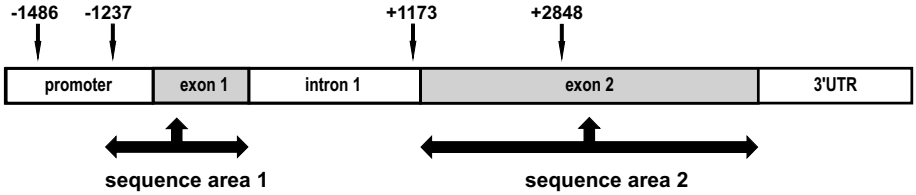

Figure 1. Schematic representation of the TLR-9 gene. The last 300 bp of the promoter together with exon1 (sequence area 1) and exon 2 (sequence area 2) were sequenced. The relative positions of the 4 SNPs studied are marked by an arrow.

Cellular stimulation of the TLR-9 pathway

In our genetic analysis we did not find differences between sarcoidosis patients and healthy controls. However, differences in transcription, translation or expression could still lead to functional differences. Therefore, in order to exclude the possibility of functional defects in the TLR-9 activation pathway in sarcoidosis patients, we tested the cytokine production upon stimulation with a TLR-9 agonist.

We found that sarcoidosis patients produce less IFN-γ upon stimulation with PHA, CpG-A DNA and CpG-A control oligonucleotide (figure 2). As expected, IFN-γ production was slightly lower in patients receiving immuno suppressive therapy in comparison with patients without these drugs (figure 3). When we measured TLR-9 induced IL-23 production a significant difference between patients and controls also was found (figure 2). In contrast to the IFN-γ data, there was no difference between patients and controls when stimulated with the CpG-A control nucleotide and only a small difference when stimulated with PHA. We did not find differences in IL-6 production upon stimulation between patients and controls indicative of a general ability in sarcoidosis patients to respond to stimuli.

Genotype		Control n=185 (%)	Study cohort					Validation cohort	
			All pts n=150 (%)	LOF n=48 (%)	A n=46 (%)	B n=36 (%)	C n=20 (%)	All pts n=383 (%)	LOF n=60 (%)
-1486	TT	54 (29.2)	50 (33.3)	16 (33.3)	16 (34.8)	10 (27.8)	8 (40.0)	135 (35.2)	18 (30.0)
	TC	100 (54.1)	72 (48.0)	25 (52.1)	21 (45.7)	20 (55.6)	6 (30.0)	186 (48.6)	35 (58.3)
	CC	31 (16.8)	28 (18.7)	7 (14.6)	9 (19.6)	6 (16.7)	6 (30.0)	62 (16.2)	7 (11.7)
-1237	TT	126 (68.1)	106 (70.7)	33 (68.8)	33 (71.7)	28 (77.8)	12 (60.0)		
	TC	55 (29.7)	42 (28.0)	15 (31.3)	12 (26.1)	8 (22.2)	7 (35.0)		
	CC	4 (2.2)	2 (1.3)	-	1 (2.2)	-	1 (5.0)		
+1173	GG	58 (31.4)	53 (35.3)	16 (33.3)	17 (37.0)	10 (27.8)	10 (50.0)		
	GA	97 (52.4)	69 (46.0)	22 (45.8)	20 (43.5)	20 (55.6)	7 (35.0)		
	AA	30 (16.2)	28 (18.7)	10 (20.8)	9 (19.6)	6 (16.7)	3 (15.0)		
+2848	AA	57 (30.8)	55 (36.7)	16 (33.3)	19 (41.3)	10 (27.8)	10 (50.0)	102 (26.6)	13 (21.7)
	AG	97 (52.4)	69 (46.0)	23 (47.9)	19 (41.3)	20 (55.6)	7 (35.0)	190 (49.6)	36 (60.0)
	GG	31 (16.8)	26 (17.3)	9 (18.8)	8 (17.4)	6 (16.7)	3 (15.0)	91 (23.8)	11 (18.3)

Table 1. Genotype distribution of the TLR-9 polymorphisms in patients and controls. Pts = patients. Radiographic evolution (over 4 years) category: LOF = Löfgren's syndrome; A = normalization or improvement towards stage 0/I; B = persistent stage II/III or progression towards stage II/III; C = stage IV at presentation or progression towards stage IV. Genotype frequencies are given in parentheses.

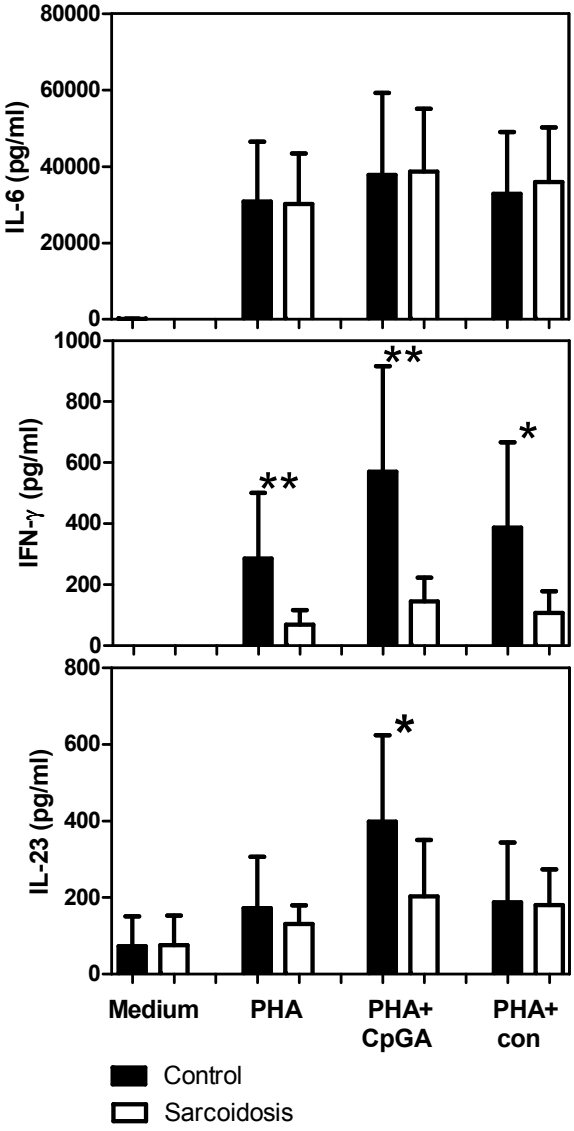

Figure 2: TLR-9 induced IL-6, IFN-γ and IL-23 production.
Mean values are shown + standard deviation. PHA=phytohaemagglutinin, CpGA=TLR-9 agonist, con= control nucleotide for CpGA. * $p<0.05$, ** $p<0.01$

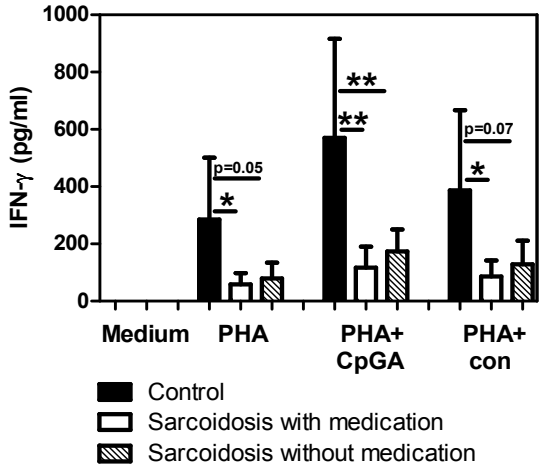

Figure 3: TLR-9 induced IFN-γ production in healthy controls and sarcoidosis patients with or without immunosuppressive therapy. Mean values are shown + standard deviation. PHA = phytohaemagglutinin, CpGA = TLR-9 agonist, con = control nucleotide for CpGA. * p<0.05, ** p<0.01.

Discussion

In the present study we investigated the genetic and functional characteristics of the innate immunity receptor TLR-9 in Dutch sarcoidosis patients. Four well known genetic polymorphisms in the TLR-9 gene were studied. We did not find an association between these polymorphisms and disease susceptibility or influence on disease course in Dutch sarcoidosis patients. The 2 polymorphisms tested in a validation cohort confirmed these data. Furthermore, to rule out genetic defects other than the known polymorphisms, the promoter region as well as exon 1 and 2 were sequenced in 20 patients. In patients with Löfgren's syndrome as well as patients with non-Löfgren sarcoidosis, no new mutations were found. Taken together, these results indicate that there is no difference in TLR-9 genetics between Dutch sarcoidosis patients and healthy controls which is in line with the recent results of Schurmann et al [8]. On the other hand, however, it is more plausible that TLR-9 agonists such as *M. tuberculosis* and *P. acnes* are triggering agents in not all, but only a fraction of sarcoidosis patients. In this subgroup of patients a genetic role

for TLR-9 could still exist. An elegant way to overcome this problem could be to subdivide patients by causative agents. In a recent study, is was estimated that 50% (n= 150) of sarcoidosis patients have T-cell memory for antigens of *M. tuberculosis* [23]. In another study it was found that 35% (n=50) of patients with sarcoidosis had an in-vitro response to a specific antigen of *P. acnes* while none of the healthy controls responded [24]. In light of antigen recognition of *M. tuberculosis* and *P. acnes* in sarcoidosis patients using these T-cell based in vitro assays, future studies in these potential subgroups of patients could reveal more insight into the role of TLR-9 polymorphisms in sarcoidosis.

In a situation where there is no genetic abnormality, functional abnormalities still could result from differences in transcription, translation or receptor expression. In order to fully explore the role of TLR-9 in sarcoidosis, it therefore is crucial to also obtain information about the function of this receptor in both patients and controls. We tested the cytokine production capacity upon stimulation with a TLR-9 agonist.

We found a decrease in IFN-γ production in sarcoidosis patients upon stimulation with a specific TLR-9 agonist. However, after stimulation with PHA, very often used as a positive control in in-vitro experiments, and the TLR-9 control nucleotide we also found a decrease in IFN-γ production. This could indicate several things. First of all, these data could reflect the anergic state of peripheral T-cells often seen sarcoidosis patients [25-28]. This anergic state could be part of the immunological paradox stating that sarcoidosis is characterized by an excessive Th-1 response, mainly at the pulmonary site, whereas circulating T-cells poorly respond to antigen challenge. Secondly, decreased IFN-γ production could also be due to a numeral disequilibrium of specific T lymphocyte subsets. In previous experiments we have demonstrated a slightly lower amount of CD4+ T cells in sarcoidosis patients indicating that this may be a contributing factor [29]. Thirdly, the genetic role of IFN-γ polymorphisms could also be of importance. We determined the allelic distribution of 3 different polymorphisms (rs2069727, rs2069718 and rs1861493) in the IFN-γ gene in the 12 patients and 12 controls used for the in vitro experiments and found no differences (data not shown). The first 2 SNPs are the most important due to their linkage disequilibrium with the functional +874 A/T polymorphism [30,31] which influences the production capacity of IFN-γ by peripheral blood mononuclear cells

and is associated with sarcoidosis [32,33]. Therefore, based on our results, genetic differences in the IFN-γ gene between patients and controls are not likely to explain the observed differences.

It is important to note that TLR-9 agonist induced IFN-γ production was impaired in both patients with and without immunosuppressive therapy (figure 3). This suggests that possible abnormalities in the TLR-9 signalling pathway are an intrinsic characteristic of sarcoidosis and not the consequence of immunosuppression.

When we measured TLR-9 induced IL-23 production we also found a significant difference between patients and controls. However, in contrast to the IFN-γ data, there was no difference between patients and controls when stimulated with the TLR-9 control nucleotide. The differences between patients and controls after stimulation with PHA were modest, and not in the order of magnitude seen with IFN-γ. This could suggest that stimulation of TLR-9 is a potent inducer of IL-23, and even more interesting, that this is somehow reduced in sarcoidosis patients, not merely based on T-cell anergy. Further investigation of the role of IL-23 in sarcoidosis is of interest due to its important role in the Th-17 pathway. It is known that *P. acnes* and *M. tuberculosis* induce both Th-1 responses as well as Th-17 responses [34,35]. It is therefore tempting to speculate that a dysregulated Th-17 response due to insufficient IL-23 production upon stimulation with either *P. acnes* or *M. tuberculosis* plays a role in the pathogenesis of sarcoidosis.

Regarding our IFN-γ and IL-23 data, it is important to state that the in vitro experiments were performed using peripheral blood monocytes and lymphocytes and not bronchoalveolar lavage (BAL) cells. It is known that the TLR expression profile in human alveolar macrophages and monocytes is not identical [36]. As mentioned earlier, sarcoidosis is characterized by an immunological paradox. Therefore, it remains to be demonstrated if the differences we found between patients and controls will also be present in the pulmonary compartment. A next step in addressing the role of TLR-9 in sarcoidosis will be to repeat our experiments using BAL monocytes and lymphocytes and include a broader range of cytokines and chemokines.

In conclusion, we demonstrate that there are no genetic differences in the gene encoding for TLR-9 between Dutch patients and healthy controls. The results from

our in vitro work demonstrate a decreased IFN-γ producing capacity in sarcoidosis patients probably based on the anergic state of peripheral T-cells. Importantly, we also found a TLR-9 specific decreased IL-23 producing capacity in sarcoidosis patients. Therefore, functional defects in the TLR-9 pathway of sarcoidosis patients could be of importance in disease susceptibility or evolution.

Reference List

1. Dubaniewicz, A., Kampfer, S. & Singh, M. Serum anti-mycobacterial heat shock proteins antibodies in sarcoidosis and tuberculosis. *Tuberculosis. (Edinb.)* **86**, 60-67 (2006).
2. Eishi, Y. *et al.* Quantitative analysis of mycobacterial and propionibacterial DNA in lymph nodes of Japanese and European patients with sarcoidosis. *J. Clin. Microbiol.* **40**, 198-204 (2002).
3. Fite, E., Fernandez-Figueras, M. T., Prats, R., Vaquero, M. & Morera, J. High prevalence of Mycobacterium tuberculosis DNA in biopsies from sarcoidosis patients from Catalonia, Spain. *Respiration* **73**, 20-26 (2006).
4. Gazouli, M. *et al.* Assessment of mycobacterial, propionibacterial, and human herpesvirus 8 DNA in tissues of greek patients with sarcoidosis. *J. Clin. Microbiol.* **40**, 3060-3063 (2002).
5. Song, Z. *et al.* Mycobacterial catalase-peroxidase is a tissue antigen and target of the adaptive immune response in systemic sarcoidosis. *J. Exp. Med.* **201**, *755-767* (2005).
6. Gazouli, M. *et al.* CARD15/NOD2, CD14, and toll-like receptor 4 gene polymorphisms in Greek patients with sarcoidosis. *Sarcoidosis. Vasc. Diffuse. Lung Dis.* **23**, 23-29 (2006).
7. Pabst, S. *et al.* Toll-like receptor (TLR) 4 polymorphisms are associated with a chronic course of sarcoidosis. *Clin. Exp. Immunol.* **143**, 420-426 (2006).
8. Schurmann, M. *et al.* Study of Toll-like receptor gene loci in sarcoidosis. *Clin. Exp. Immunol.* **152**, 423-431 (2008).
9. Tanabe, T. *et al.* Sarcoidosis and NOD1 variation with impaired recognition of intracellular Propionibacterium acnes. *Biochim. Biophys. Acta* **1762**, *794-801* (2006).
10. Veltkamp, M., Grutters, J. C., van Moorsel, C. H., Ruven, H. J. & van den Bosch, J. M. Toll-like receptor (TLR) 4 polymorphism Asp299Gly is not associated with disease course in Dutch sarcoidosis patients. *Clin. Exp. Immunol.* **145**, 215-218 (2006).
11. Veltkamp, M. *et al.* CD14 genetics in sarcoidosis patients; who's in control? *Sarcoidosis. Vasc. Diffuse. Lung Dis.* **24**, 154-155 (2007).
12. Veltkamp, M. *et al.* Linkage between Toll-like receptor (TLR) 2 promotor and intron polymorphisms: functional effects and relevance to sarcoidosis. *Clin. Exp. Immunol.* **149**, 453-462 (2007).
13. Schurmann, M. *et al.* Results from a genome-wide search for predisposing genes in sarcoidosis. *Am. J. Respir. Crit Care Med.* **164**, 840-846 (2001).
14. Kalis, C. *et al.* Requirement for TLR9 in the immunomodulatory activity of Propionibacterium acnes. *J Immunol. 2005 Apr* **1;174**, 4295-4300.
15. Bafica, A. *et al.* TLR9 regulates Th1 responses and cooperates with TLR2 in mediating optimal resistance to Mycobacterium tuberculosis. *J Exp Med. 2005 Dec* **19;202**, 1715-1724.

16. Hornung, V. *et al.* Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J Immunol. 2002 May* **1;168**, 4531-4537.

17. Grutters, J. C. *et al.* Analysis of IL6 and IL1A gene polymorphisms in UK and Dutch patients with sarcoidosis. *Sarcoidosis Vasc Diffuse Lung Dis 2003 Mar;20(1):20-7. -7*.

18. Lazarus, R. *et al.* Single-nucleotide polymorphisms in the Toll-like receptor 9 gene (TLR9): frequencies, pairwise linkage disequilibrium, and haplotypes in three U.S. ethnic groups and exploratory case-control disease association studies. *Genomics* **81**, 85-91 (2003).

19. Novak, N. *et al.* Putative association of a TLR9 promoter polymorphism with atopic eczema. *Allergy* **62**, 766-772 (2007).

20. Bunce, M. *et al.* Phototyping: comprehensive DNA typing for HLA-A, B, C, DRB1, DRB3, DRB4, DRB5 & DQB1 by PCR with 144 primer mixes utilizing sequence-specific primers (PCR-SSP). *Tissue Antigens 1995 Nov;46(5):355-67. -67*.

21. de Jager, W. & Rijkers, G. T. Solid-phase and bead-based cytokine immunoassay: a comparison. *Methods* **38**, 294-303 (2006).

22. de Jager, W., Prakken, B. & Rijkers, G. T. Cytokine multiplex immunoassay: methodology and (clinical) applications. *Methods Mol. Biol.* **514**, 119-133 (2009).

23. Chen, E. S. *et al.* T cell responses to mycobacterial catalase-peroxidase profile a pathogenic antigen in systemic sarcoidosis. *J. Immunol.* **181**, 8784-8796 (2008).

24. Ebe, Y. *et al.* Proliferative response of peripheral blood mononuclear cells and levels of antibody to recombinant protein from Propionibacterium acnes DNA expression library in Japanese patients with sarcoidosis. *Sarcoidosis. Vasc. Diffuse. Lung Dis.* **17**, 256-265 (2000).

25. Bertran, G., Arzt, E., Resnik, E., Mosca, C. & Nahmod, V. Inhibition of interferon gamma production by peripheral blood mononuclear leukocytes of patients with sarcoidosis. Pathogenic implications. *Chest* **101**, 996-999 (1992).

26. Daniele, R. P., Dauber, J. H. & Rossman, M. D. Immunologic abnormalities in sarcoidosis. *Ann. Intern. Med.* **92**, 406-416 (1980).

27. Goodwin, J. S., DeHoratius, R., Israel, H., Peake, G. T. & Messner, R. P. Suppressor cell function in sarcoidosis. *Ann. Intern. Med.* **90**, 169-173 (1979).

28. Rottoli, P., Muscettola, M., Grasso, G., Perari, M. G. & Vagliasindi, M. Imparied interferon-gamma production by peripheral blood mononuclear cells and effects of calcitriol in pulmonary sarcoidosis. *Sarcoidosis.* **10**, 108-114 (1993).

29. Heron, M., Claessen, A. M., Grutters, J. C. & van den Bosch, J. M. T cell activation profiles in different granulomatous interstitial lung diseases - a role for CD8(+) CD28(null) cells? *Clin. Exp. Immunol.* (2009).

30. Kantarci, O. H. *et al.* Interferon gamma allelic variants: sex-biased multiple sclerosis susceptibility and gene expression. *Arch. Neurol.* **65**, 349-357 (2008).

31. Kim, K. *et al.* Interferon-gamma gene polymorphisms associated with susceptibility to systemic lupus erythematosus. *Ann. Rheum. Dis.* (2009).

32. Pravica, V., Perrey, C., Stevens, A., Lee, J. H. & Hutchinson, I. V. A single nucleotide polymorphism in the first intron of the human IFN-gamma gene: absolute correlation with a polymorphic CA microsatellite marker of high IFN-gamma production. *Hum. Immunol.* **61**, 863-866 (2000).

33. Wysoczanska, B., Bogunia-Kubik, K., Suchnicki, K., Mlynarczewska, A. & Lange, A. Combined association between IFN-gamma 3,3 homozygosity and DRB1*03 in Lofgren's syndrome patients. *Immunol. Lett.* **91**, 127-131 (2004).

34. Miossec, P., Korn, T. & Kuchroo, V. K. Interleukin-17 and type 17 helper T cells. *N. Engl. J. Med.* **361**, 888-898 (2009).

35. Zenaro, E., Donini, M. & Dusi, S. Induction of Th1/Th17 immune response by Mycobacterium tuberculosis: role of dectin-1, Mannose Receptor, and DC-SIGN. *J. Leukoc. Biol.* **86**, 1393-1401 (2009).

36. Juarez, E. *et al.* Differential expression of Toll-like receptors on human alveolar macrophages and autologous peripheral monocytes. *Respir. Res.* **11**, 2 (2010).

6

Genetic variation in the Toll-like Receptor gene cluster (TLR10-TLR1-TLR6) influences disease course in sarcoidosis

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Abstract

Sarcoidosis is an inflammatory disease of unknown etiology. Various micro-organisms have been proposed as etiologic agent suggesting a role for pattern-recognition receptors such as Toll-like receptors (TLR) in disease pathogenesis, with a special interest in TLR-2. TLR-10, TLR-1 and TLR-6 all can act as a co-receptor for TLR-2 and the genes encoding these receptors are located in a gene cluster on chromosome 4. The aim of our study was to assess differences in genetic variation in the TLR10-TLR1-TLR6 gene cluster between patients and controls. A total of 8 SNPs were genotyped in 447 healthy controls and 533 patients, divided in 425 with sarcoidosis and 108 with Löfgren's syndrome. Comparison of the total patient cohort with controls showed that the allele frequencies of rs1109695, rs7658893 (TLR10) and rs5743604 as well as rs5743594 (TLR1) differed significantly. Haplotype analysis revealed that the most common haplotype found was significantly decreased in patients with chronic sarcoidosis. In patients with Löfgren's syndrome another haplotype also demonstrated a decreased prevalence compared to healthy controls. Finally, a less common haplotype was found to be significantly increased in patients with Löfgren's syndrome as well as sarcoidosis patients with self-remitting disease, indicating that it could act as a disease modifying haplotype.

In conclusion, our study suggests that absence of the common haplotype in the TLR10-TLR1-TLR6 gene cluster increases the risk of developing chronic disease in patients already affected by sarcoidosis. Two other haplotypes in the TLR10-TLR1-TLR6 gene cluster predispose to Löfgren's syndrome and a self-remitting disease course in sarcoidosis, indicating that it could also be a disease modifying haplotype. Based on their role as co-receptors for TLR-2, this study supports the growing evidence that aberrant TLR-2 function is important in sarcoidosis disease pathogenesis and could also be of influence in patients with Löfgren's syndrome.

Introduction

Sarcoidosis is a systemic disorder characterized by the formation of non-caseating granulomas, primarily affecting lymph nodes and lungs. Given the variation in clinical spectrum, it is suggested that sarcoidosis reflects a collection of different granulomatous diseases, each with its own inducing antigen. Immunologically, the disease is characterized by a strong Thelper-1 (Th1) immune response, suggesting a role for intracellular pathogens. Due to partial clinical, radiological and pathological resemblance with tuberculosis, many studies have focussed on the potential role of *Mycobacterium tuberculosis* in the etiology and pathogenesis of sarcoidosis [1-5]. Another intracellular bacterium, the commensal *Propionibacterium acnes* has also been extensively studied. Isolation of *P. acnes* from sarcoid lesions [6], presence of *P. acnes* DNA in sarcoid lymph nodes [7] and studies in mice [8,9] all suggest an etiological link between *P. acnes* and some cases of sarcoidosis.

In recent years it has been established that the innate immune system is of prime importance for the onset and regulation of the adaptive immune response. Is also has become clear that the innate immune system isn't non-specific but able to recognize molecular patterns of micro-organisms. An important category of innate immunity receptors serving this function are the Toll-like receptors (TLR), a family of related transmembrane or endosomal molecules each recognizing a distinct, but limited, repertoire of microbial encoded molecules. Of the various different TLRs, TLR-2, recognizing peptidoglycans, lipopeptides and heat shock proteins, has been suggested to play a role in the pathogenesis of sarcoidosis [10-12]. Interestingly, TLR-2 is important in the innate immune response against both *Mycobacterium tuberculosis* as well as *Propionibacterium acnes* [13-16]. In previous work, our group demonstrated an increased prevalence of the AA genotype of the TLR2 promoter polymorphism – 16934 A/T in sarcoidosis patients with a chronic course. However, this association could not be replicated in a second cohort of Dutch patients [17]. As indicated above, TLR-2 can interact with a variety of microbial components and it has been shown that TLR-1 and TLR-6 are co-receptors for TLR-2. Heterodimers of either TLR-2/1 or TLR-2/6 have different agonist specificities[18]. Recently, it has been suggested that TLR-10 also functions as a co-receptor for TLR-2 [19]. TLR1, TLR6 and TLR10 are clustered

in a 54-kb region on chromosome 4p14 and encode proteins that share a high degree of homology in their overall amino acid sequences. Taken together, when considering a causative role for intracellular pathogens such as *M. tuberculosis* and *P. acnes* in the pathogenesis of sarcoidosis, the Toll-like Receptors TLR-1, TLR-6 and TLR-10, based on their function as co-receptors for TLR-2, are attractive candidates for genetic analysis in sarcoidosis research. We hypothesize that genetic variations in these genes may result in a different TLR-2 function contributing to an aberrant immune response. Therefore, we postulate that certain genotypes of SNPs located in the TLR10-TLR1-TLR6 gene cluster are more prevalent in sarcoidosis patients than in the general population.

Materials and Methods

Study subjects for genetic analysis

533 unrelated Dutch Caucasian patients presenting with sarcoidosis at the St. Antonius Hospital (259 women and 274 men) were included in this study. In 425 patients, the diagnosis of sarcoidosis was established when clinical findings were supported by histological evidence, and after the exclusion of other known causes of granulomatosis. 108 patients presented with the classical characteristics of Löfgren's syndrome; fever, erythema nodosum, bilateral hilar lymphadenopathy and joint symptoms. The control subjects comprised 447 healthy, Dutch Caucasian employees of the St Antonius Hospital and blood samples from Sanquin blood bank in the Netherlands (202 women and 245 men). By completing a questionnaire, relevant background information was provided by these volunteers and included medication, ethnicity and hereditary diseases. Verbal and written consent was obtained from all subjects, and authorization was given by the Ethics Committee of the St. Antonius Hospital, Nieuwegein.

Radiographic staging

Pulmonary disease severity of sarcoidosis patients at presentation was evaluated by chest radiography and pulmonary function testing. Longitudinal chest radiographs for each patient were examined and compared to determine disease outcome. Chest radiographs were collected for each patient and assessed blind by a pulmonary physician for disease severity using standard radiographic staging for sarcoidosis. In brief, this comprises five stages: stage 0: normal, stage I: bilateral hilar lymphadenopathy (BHL), stage II: BHL and parenchymal infiltration, stage III: parenchymal infiltration without BHL, stage IV: irreversible fibrosis with loss of lung volume. Radiographic evolution over a minimal 4 year period was available for 276 patients and was categorized as follows: A (normalization of improvement towards stage I), B (persistent stage II/III or progression into that direction, and C (stable stage IV or progression towards this stage) [20]. Patients presenting with pulmonary fibrosis or showing pulmonary fibrosis on chest radiography at follow-up less than 4 years after diagnosis were classified as stage IV at disease outcome considering the irreversibility of fibrotic scarring. Patients who have been diagnosed with Löfgren's syndrome at presentation were considered as a distinct group with radiographic evolution not exceeding stage I.

Analysis of genetic polymorphisms

DNA was extracted from whole blood samples and SNPs were analyzed on a custom goldengate bead SNP assay that was performed in accordance with the manufacturer's recommendations (Illumina Inc, San Diego, USA). A total of 8 SNPs were selected based on functionality, minor allele frequency >0.10 and location in order to allow haplotypes analysis (figure 1).

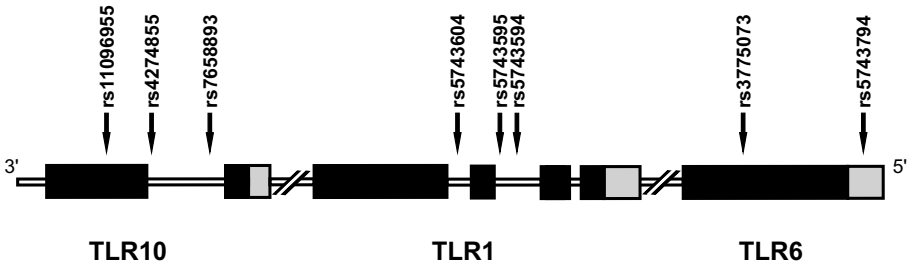

Figure 1. Schematic representation of TLR10-TLR1-TLR-6 gene cluster on chromosome 4. Relative positions of the 8 single nucleotide polymorphisms studied are marked by an arrow. Grey bar = promoter region, parallel horizontal lines = intron, black bar = exon. An A to C nucleotide substitution at location rs11096955 (TLR10, exon 2) leads to the replacement of Isoleucine to Leucine at amino acid location 369 in the extracellular domain of the receptor. An A to G nucleotide substitution at location rs3775073 (exon 1 TLR6) does not change the Lysine at amino acid location 421.

Haplotype determinations

Linkage between the different SNPs was determined using Haploview version 4.0 [21]. Individual haplotypes were inferred using PHASEv2 software. This program uses an advanced algorithm based on a method by Stephens et al [22].

Statistical analysis

Allele and genotype frequencies were calculated for each locus and tested for Hardy-Weinberg equilibrium in controls. Association studies were analysed by chi-square testing using 2x3 and 2x2 contingency tables for genotype and allele frequencies, respectively. P-values were corrected using a Bonferroni correction for multiple testing. A p value <0.05 was considered significant.

Results

Distribution of TLR10-TLR1-TLR6 gene variation in patients and healthy controls

Table 1 summarizes the allele and genotype frequencies of the investigated TLR10-TLR1-TLR6 SNPs in sarcoidosis patients (including patients with Löfgren's syndrome) and healthy controls. The SNPs did not deviate from Hardy-Weinberg equilibrium in controls. No full linkage disequilibrium was observed. High linkage disequilibrium values of r^2 were observed between rs4274855, rs5743595, and rs5743794 ,located in TLR10, TLR1, and TLR6 respectively (figure 2). When comparing all patients with controls, allele frequencies from rs1109695, rs7658893 (TLR10) and rs5743604 as well as rs5743594 (TLR1) differed significantly, even after correction for multiple testing (table 1). The allele frequencies of these 4 SNPs did not differ between sarcoidosis patients and patients with Löfgren's syndrome, indicating an effect in both patient groups. However, because of the relative small number of Löfgren's patients, the associations lost significance after Bonferroni correction in this group. To assess a possible influence of the SNPs mentioned above on disease course, allele frequencies of all 8 SNPs were calculated in sarcoidosis patients with self-limiting disease and patients with a chronic course of sarcoidosis. No significant differences were found between patients with self-limiting disease and chronic sarcoidosis, however the allele frequencies of rs1109695, rs7658893 (TLR10) and rs5743604 (TLR1) in chronic patients differed significantly from healthy controls (table 2). There were no differences in allele frequencies between chronic patients with pulmonary fibrosis and chronic patients without pulmonary fibrosis.

SNP	Allele	Control	Sarcoidosis	Löfgren	Genotype	Control	Sarcoidosis	Lofgren	P value [$P_{corrected}$] Ctr vs Pts	Ctr vs Sar	Ctr vs Löf
TLR10											
rs11096955	A	0.65	0.58	0.57	AA	0.42	0.31	0.35	0.0007 **[0.002]**	0.002 **[0.005]**	0.0327 [ns]
	C	0.35	0.42	0.43	AC	0.47	0.54	0.44			
					CC	0.11	0.15	0.21			
rs4274855	A	0.18	0.19	0.21	AA	0.04	0.04	0.03	0.47 [ns]	0.66 [ns]	0.32 [ns]
	G	0.82	0.81	0.79	AG	0.27	0.30	0.36			
					GG	0.68	0.66	0.60			
rs7658893	A	0.24	0.31	0.31	AA	0.06	0.09	0.08	0.0004 **[0.001]**	0.0006 **[0.002]**	0.05 [ns]
	G	0.76	0.69	0.69	AG	0.36	0.44	0.45			
					GG	0.58	0.47	0.47			
TLR1											
rs5743604	A	0.75	0.68	0.69	AA	0.57	0.47	0.48	0.001 **[0.003]**	0.002 **[0.005]**	0.08 [ns]
	G	0.25	0.32	0.31	AG	0.37	0.42	0.42			
					GG	0.06	0.11	0.10			
rs5743595	A	0.81	0.80	0.79	AA	0.68	0.64	0.61	0.38 [ns]	0.45 [ns]	0.50 [ns]
	G	0.19	0.20	0.21	AG	0.28	0.32	0.36			
					GG	0.05	0.04	0.03			
rs5743594	A	0.20	0.16	0.15	AA	0.05	0.02	0.04	0.016 **[0.049]**	0.03 [ns]	0.1 [ns]
	G	0.80	0.84	0.85	AG	0.29	0.27	0.22			
					GG	0.65	0.70	0.74			
TLR6											
rs3775073	A	0.68	0.64	0.67	AA	0.45	0.41	0.42	0.15 [ns]	0.12 [ns]	0.8 [ns]
	G	0.32	0.36	0.33	AG	0.46	0.46	0.5			
					GG	0.09	0.13	0.08			
rs5743794	A	0.20	0.21	0.21	AA	0.05	0.04	0.02	0.75 [ns]	0.81 [ns]	0.78 [ns]
	G	0.80	0.79	0.79	AG	0.30	0.34	0.39			
					GG	0.65	0.62	0.59			

Table 1. Allele and genotype frequencies of TLR10, TLR1 and TLR6 polymorphisms in patients and controls. Ctr = controls. Pts = patients. Sarcoidosis = patients without Löfgren's syndrome. Löf = patients with Löfgren's syndrome. P values are based on allele frequency differences. P values corrected for multiple testing (Bonferroni correction) are shown between brackets. Significant values are printed in bold. ns = non significant. Cumulative frequencies do not add up to 1.0 due to rounding.

			Clinical phenotype		P value [$p_{corrected}$]	
SNP	**Allele**	**Control**	**A**	**BC**	**A vs BC**	**Control vs BC**
TLR10						
rs11096955	A	0.65	0.62	0.55	0.16 [ns]	0.008 **[0.02]**
	C	0.35	0.38	0.45		
rs7658893	A	0.24	0.28	0.35	0.1 [ns]	0.0007 **[0.002]**
	G	0.76	0.72	0.65		
TLR1						
rs5743604	A	0.75	0.72	0.64	0.08 [ns]	0.001 **[0.003]**
	G	0.25	0.28	0.36		
rs5743594	A	0.20	0.19	0.16	0.32 [ns]	0.14 [ns]
	G	0.80	0.81	0.84		

Table 2. Allele frequencies of 4 polymorphisms in TLR10 and TLR1 predisposing for sarcoidosis in patients with self-remitting and chronic sarcoidosis. Clinical phenotype based on radiographic evolution over a minimum 4 year period. A = normalization of improvement towards stage I (n=154), B = persistent stage II/III or progression in that direction (n=61) and C = stable stage IV or progression towards this stage (n=61). BC = patients with persistent stage II/III/IV or progression in that direction (n=122). vs. = versus. P values corrected for multiple testing (Bonferroni correction) are shown between brackets. Significant values are printed in bold. ns = non significant.

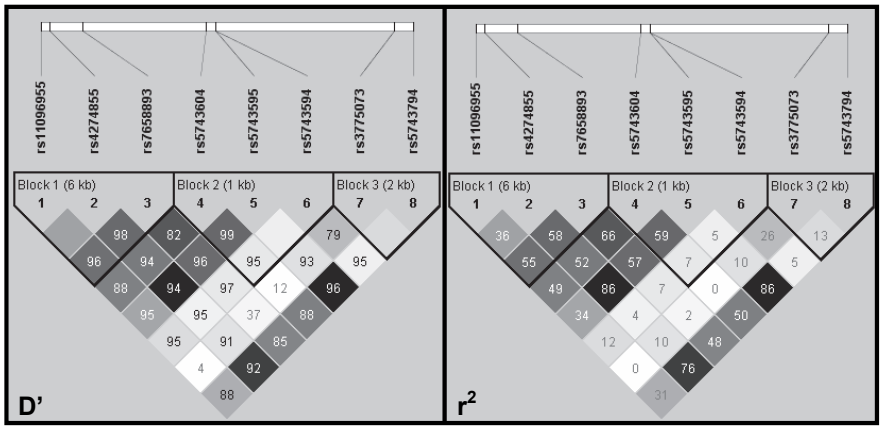

Figure 2. Pair-wise linkage disequilibrium values of TLR10-TLR1-TLR6 single-nucleotide polymorphisms in a Dutch population. Values of the pair-wise D' (x100) and r^2 (x100) are shown in blocks.

Distribution of TLR10-TLR1-TLR6 haplotypes in patients and healthy controls

The construction of haplotypes based on 8 SNPs produced a total of 32 different haplotypes of which five had a frequency >5% in at least one of the populations. These haplotypes are numbered 1 to 5 and shown in table 3 with their observed frequencies. The frequency of haplotype 1 was significantly lower in sarcoidosis patients as compared to healthy controls. Separate analysis of patients with a self-remitting or chronic disease course revealed that the significant difference could be attributed to a lower frequency of haplotype 1 in patients with chronic disease. The frequency of haplotype 3 was significantly lower in patients with Löfgren's syndrome. Haplotype 5 is rare in controls (3%), but twice as prevalent in both patients with a self-remitting disease course of sarcoidosis and Löfgren's syndrome (6%, $p<0.05$).

Haplo type	SNP position								Controls	Sarcoidosis	Löfgren	Sarcoidosis		P value		
	TLR10			TLR1			TLR6					A	BC	Ctr vs. S	Ctr vs. L	Ctr vs. BC[a] Ctr vs. A[b]
	rs11096955	rs4274855	rs7658893	rs5743604	rs5743595	rs5743594	rs3775073	rs5743794								
1	A	G	G	A	A	G	A	G	0.42(367)	0.37(295)	0.41(80)	0.38(110)	0.30(50)	**0.04**	0.9	**0.008[a]**
2	C	A	A	G	G	G	A	A	0.20(173)	0.19(151)	0.19(37)	0.16(46)	0.19(31)	0.68	0.9	
3	A	G	G	A	A	A	G	G	0.17(147)	0.14(115)	0.10(20)	0.17(50)	0.16(27)	0.2	0.03	
4	C	G	G	A	A	G	G	G	0.05(48)	0.06(45)	0.08(16)	0.07(19)	0.08(13)	1	0.2	
5	C	G	A	G	A	G	G	G	0.03(26)	0.06(46)	0.06(12)	0.06(17)	0.04(6)	**0.008**	**0.048**	**0.034[b]**
6-32	-	-	-	-	-	-	-	-	0.13(115)	0.19(150)	0.15(29)	0.17(48)	0.35(57)			

Table 3. TLR10-TLR1-TLR6 haplotype frequencies in controls, sarcoidosis (including self-remitting compared to chronic disease) and Löfgren patients. Frequencies are calculated from the number of alleles. Absolute numbers are in parentheses. Clinical phenotypes are based on radiographic evolution over a minimum 4 year period. A = normalization of improvement towards stage I (n=154), B = persistent stage II/III or progression in that direction (n=61) and C = stable stage IV or progression towards this stage (n=61). vs. = versus. Ctr vs. S = Controls vs. Sarcoidosis. Ctr vs. L = Controls vs. patients with Löfgren's syndrome. Ctr vs A = Controls vs. Sarcoidosis patients with self-remitting disease, Ctr vs BC means Controls vs. Sarcoidois patients with chronic course (with and without fibrosis). P values were calculated using 2x2 contingency tables. Significant values are printed in bold. Cumulative frequencies sometimes do not add up to 1.0 due to rounding.

Discussion

In the present study we investigated 8 different single nucleotide polymorphisms (SNPs) located in the TRL10-TLR1-TLR6 gene cluster. Four of these SNPs were found to be associated with either sarcoidosis or Löfgren's syndrome. Three of these SNPs, two in TLR10 and one in TLR1, had a decrease of major allele frequency in both sarcoidosis patients as well as patients with Löfgren's syndrome, although the limited number of patients prevented it to reach statistical significance in the latter group. When sarcoidosis patients with a self-remitting or chronic disease course were analyzed separately it was observed that the differences found for the three SNPs are due to a low prevalence of the major alleles in chronic sarcoidosis (table 2). The increase of the major allele of rs5743594 (TLR1) was observed in both patients with chronic sarcoidosis as well as Löfgren's syndrome but did not withstand Bonferroni correction for multiple testing in the latter group.

Although linkage disequilibrium was high between three SNPs in the gene cluster, the SNPs associating with susceptibility and course of the disease were not in linkage with each other. For the 3 intron SNPs associated with sarcoidosis and Löfgren's syndrome, we must consider the possibility that other SNPs that were not genotyped in our study but that are in linkage disequilibrium with these variants could be responsible for functional differences in the Toll-like receptors.

Haplotypes were constructed using all 8 SNPs investigated in this study (table 3). Association analysis revealed that the prevalence of the most common haplotype found was significantly decreased in sarcoidosis patients but not in patients with Löfgren's syndrome. Once again, separate analysis of patients with self-remitting or a chronic disease course showed that this was due to a decreased prevalence in chronic sarcoidosis. The allele of the TLR10 exon SNP rs11096955 at position 1 of haplotype 1 was the A allele in concordance with the SNP analysis. This finding could indicate that the exon SNP in TLR10 can solely influence disease course in sarcoidosis. An A to C nucleotide substitution of rs11096955 leads to the replacement of Isoleucine to Leucine in the extracellular domain of the TLR-10 receptor. This could result in differential ligand binding capacity for its ligand, but functional studies have not been published at the present time. It could be

speculated that differences in binding capacity of TLR-10 could influence the function of the TLR-2/10 heterodimer in sarcoidosis. In other words, absence of the major allele of this SNP in patients who already have sarcoidosis may increase the risk of developing a chronic course. On the other hand, the decreased prevalence of a haplotype covering almost the entire TLR10-TLR1-TLR6 gene cluster can also indicate that allelic differences of SNPs divided over all three genes play a role in disease pathogenesis. TLR10 and TLR6 gene polymorphisms have been associated with an increased risk of prostate cancer and asthma [23-25]. More interesting with regard to sarcoidosis pathogenesis, is the finding that the exon SNP rs4933095 (N248S) in TLR1 is associated with an increased TLR-1 expression, aberrant mycobacterial signaling [26,27] and tuberculosis disease susceptibility [28]. This N248S polymorphism has a high degree of linkage disequilibrium with polymorphism rs5743595 tested in our study (D'=0.99 and r^2=0.76, personal communication Mrs V. Stevens [25]). The decreased prevalence of haplotype 1 in patients with chronic sarcoidosis could therefore also suggest that genetic variation in TLR10, TLR1 as well as TLR6 can play a role in disease pathogenesis. The data implicating that genetic variation in the TLR10-TLR1-TLR6 gene cluster can influence disease course are even more interesting when compared with known TLR2 polymorphisms in sarcoidosis. In previous work, our group demonstrated an increased prevalence of the AA genotype of the TLR2 promoter polymorphism – 16934 A/T in sarcoidosis patients with a chronic disease course [17]. Although not reproduced in a validation cohort, the presence of this genotype resulted in a higher amount of TNF-alpha production upon stimulation with TLR-2 agonists which could partly explain the increased amount of TNF-alpha often found in chronic sarcoidosis. The fact that genetic variation of the TLR10-TLR1-TLR6 gene cluster is also found in patients with chronic disease strengthens, in our opinion, the hypothesis that an aberrant function of TLR-2 predisposes for developing chronic disease in patients already affected by sarcoidosis.

In patients with Löfgren's syndrome, a lower prevalence of haplotye 3 was found suggesting that this haplotype has a protective effect against developing Löfgren's syndrome. Furthermore, an increased occurrence of haplotype 5 also was found suggesting a role in disease susceptibility as well. Interestingly, however, this haplotype had a significantly higher prevalence in patients with a self-remitting form

of sarcoidosis. This could implicate that it is not a disease susceptibility haplotype, but a disease modifying one in favor of a good prognosis in both sarcoidosis and Löfgren's syndrome. No associations between Toll-like receptors and Löfgren's syndrome have previously been described in literature. However, genetic polymorphisms in the gene encoding for CD14, capable of forming a complex with TLR-2 as well, already have been associated with Löfgren's syndrome [29] suggesting a possible role for TLR-2 in Löfgren disease pathogenesis.

In summary, our study suggests that absence of the most common haplotype in the TLR10-TLR1-TLR6 gene cluster increases the risk of developing chronic disease in patients already affected by sarcoidosis. Two other haplotypes in the TLR10-TLR1-TLR6 gene cluster could predispose for developing Löfgren's syndrome or influence disease course. Based on their role as co-receptors for TLR-2, this study supports the growing evidence that aberrant TLR-2 function plays a role in sarcoidosis disease pathogenesis and could also be of influence in patients with Löfgren's syndrome.

Reference List

1. Chen, E. S. *et al.* T cell responses to mycobacterial catalase-peroxidase profile a pathogenic antigen in systemic sarcoidosis. *J. Immunol.* **181**, 8784-8796 (2008).
2. Gupta, D., Agarwal, R., Aggarwal, A. N. & Jindal, S. K. Molecular evidence for the role of mycobacteria in sarcoidosis: a meta-analysis. *Eur. Respir. J.* **30**, 508-516 (2007).
3. Oswald-Richter, K. *et al.* Mycobacterial ESAT-6 and katG are recognized by sarcoidosis CD4+ T cells when presented by the American sarcoidosis susceptibility allele, DRB1*1101. *J. Clin. Immunol.* **30**, 157-166 (2010).
4. Oswald-Richter, K. A. *et al.* Cellular responses to mycobacterial antigens are present in bronchoalveolar lavage fluid used in the diagnosis of sarcoidosis. *Infect. Immun.* **77**, 3740-3748 (2009).
5. Song, Z. *et al.* Mycobacterial catalase-peroxidase is a tissue antigen and target of the adaptive immune response in systemic sarcoidosis. *J. Exp. Med.* **201**, 755-767 (2005).
6. Abe, C., Iwai, K., Mikami, R. & Hosoda, Y. Frequent isolation of Propionibacterium acnes from sarcoidosis lymph nodes. *Zentralbl. Bakteriol. Mikrobiol. Hyg. A* **256**, 541-547 (1984).
7. Eishi, Y. *et al.* Quantitative analysis of mycobacterial and propionibacterial DNA in lymph nodes of Japanese and European patients with sarcoidosis. *J. Clin. Microbiol.* **40**, 198-204 (2002).
8. Iio, K. *et al.* Experimental pulmonary granuloma mimicking sarcoidosis induced by Propionibacterium acnes in mice. *Acta Med. Okayama* **64**, 75-83 (2010).
9. McCaskill, J. G. *et al.* Pulmonary immune responses to Propionibacterium acnes in C57BL/6 and BALB/c mice. *Am. J. Respir. Cell Mol. Biol.* **35**, 347-356 (2006).
10. Chen, E. S. *et al.* Serum amyloid A regulates granulomatous inflammation in sarcoidosis through Toll-like receptor-2. *Am. J. Respir. Crit Care Med.* **181**, 360-373 (2010).
11. Oswald-Richter, K. A. *et al.* Cellular responses to mycobacterial antigens are present in bronchoalveolar lavage fluid used in the diagnosis of sarcoidosis. *Infect. Immun.* **77**, 3740-3748 (2009).
12. Wiken, M., Grunewald, J., Eklund, A. & Wahlstrom, J. Higher monocyte expression of TLR2 and TLR4, and enhanced pro-inflammatory synergy of TLR2 with NOD2 stimulation in sarcoidosis. *J. Clin. Immunol.* **29**, 78-89 (2009).
13. Bowdish, D. M. *et al.* MARCO, TLR2, and CD14 are required for macrophage cytokine responses to mycobacterial trehalose dimycolate and Mycobacterium tuberculosis. *PLoS. Pathog.* **5**, e1000474 (2009).
14. Jugeau, S. *et al.* Induction of toll-like receptors by Propionibacterium acnes. *Br. J. Dermatol.* **153**, 1105-1113 (2005).

15. Romics, L., Jr. *et al.* Toll-like receptor 2 mediates inflammatory cytokine induction but not sensitization for liver injury by Propioni- bacterium acnes. *J. Leukoc. Biol.* **78**, 1255-1264 (2005).

16. Yim, J. J. *et al.* The association between microsatellite polymorphisms in intron II of the human Toll-like receptor 2 gene and tuberculosis among Koreans. *Genes Immun.* **7**, 150-155 (2006).

17. Veltkamp, M. *et al.* Linkage between Toll-like receptor (TLR) 2 promotor and intron polymorphisms: functional effects and relevance to sarcoidosis. *Clin. Exp. Immunol.* **149**, 453-462 (2007).

18. Kirschning, C. J. & Schumann, R. R. TLR2: cellular sensor for microbial and endogenous molecular patterns. *Curr. Top. Microbiol. Immunol.* **270**, 121-144 (2002).

19. Guan, Y. *et al.* Human TLRs 10 and 1 share common mechanisms of innate immune sensing but not signaling. *J. Immunol.* **184**, 5094-5103 (2010).

20. Grutters, J. C. *et al.* Analysis of IL6 and IL1A gene polymorphisms in UK and Dutch patients with sarcoidosis. *Sarcoidosis Vasc Diffuse Lung Dis 2003 Mar;20(1):20-7. -7*.

21. Barrett, J. C., Fry, B., Maller, J. & Daly, M. J. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics.* **21**, 263-265 (2005).

22. Stephens, M. & Donnelly, P. A comparison of bayesian methods for haplotype reconstruction from population genotype data. *Am. J. Hum. Genet.* **73**, 1162-1169 (2003).

23. Lazarus, R. *et al.* TOLL-like receptor 10 genetic variation is associated with asthma in two independent samples. *Am. J. Respir. Crit Care Med* **170**, 594-600 (2004).

24. Kormann, M. S. *et al.* Toll-like receptor heterodimer variants protect from childhood asthma. *J. Allergy Clin. Immunol.* **122**, 86-92, 92 (2008).

25. Stevens, V. L. *et al.* Genetic variation in the toll-like receptor gene cluster (TLR10-TLR1-TLR6) and prostate cancer risk. *Int. J. Cancer* **123**, 2644-2650 (2008).

26. Hawn, T. R. *et al.* A common human TLR1 polymorphism regulates the innate immune response to lipopeptides. *Eur. J. Immunol.* **37**, 2280-2289 (2007).

27. Misch, E. A. *et al.* Human TLR1 deficiency is associated with impaired mycobacterial signaling and protection from leprosy reversal reaction. *PLoS. Negl. Trop. Dis* **2**, e231 (2008).

28. Ma, X. *et al.* Full-exon resequencing reveals toll-like receptor variants contribute to human susceptibility to tuberculosis disease. *PLoS. One.* **2**, e1318 (2007).

29. Veltkamp, M. *et al.* CD14 genetics in sarcoidosis patients; who's in control? *Sarcoidosis. Vasc. Diffuse. Lung Dis.* **24**, 154-155 (2007).

7

In vitro γ-interferon responses to *Mycobacterium tuberculosis*, *Propionibacterium acnes* and *P. granulosum* in Dutch sarcoidosis patients

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Abstract

Mycobacterium tuberculosis and both *Propionibacterium acnes* and *Propionibacterium granulosum* have been proposed as candidate causative agents for sarcoidosis, a systemic granulomatous disease. The aim of this study was to assess the T-cell responses against these 3 bacteria in Dutch sarcoidosis patients. The commercially available Tspot-TB® was used to detect latent tuberculosis infection in 57 sarcoidosis patients and tested positive in 3 patients (5.3%). Furthermore, an enzyme-linked immunospot assay (ELISPOT) was developed using *P. acnes* or *P. granulosum* as antigen stimulation and phytohaemagglutinin (PHA) as a positive control. A T-cell response against both propionibacteria was found in all healthy controls as well as sarcoidosis patients. A lower but non-significant difference was found in the number of spots induced by PHA between patients and controls ($p=0.06$), suggesting a decreased IFN-γ producing capacity in peripheral T cells of patients. No differences were found in amount of spots induced by both propionibacteria between patients and controls. A correlation between the amount of spots induced by PHA and *P. acnes* as well as *P. granulosum* was found ($p=0.004$ and $p=0.003$, respectively) indicating some mitogenic activity for both bacteria. In conclusion, our data do not provide strong linkage between sarcoidosis and previous infection with *M. tuberculosis* in The Netherlands. Comparing responses to propionibacteria was hampered by the observations that sarcoidosis patients have an overall decreased IFN-γ producing capacity and the suggested mitogenic capacity of these bacteria. Importantly, the suggested mitogenic activity of propionibacteria provides new insights on how these commensal bacteria can play a role in sarcoidosis disease pathogenesis.

Introduction

Sarcoidosis is a systemic disease characterized by non-caseating epithelioid granulomatous inflammation with pulmonary and lymph node involvement in most patients. There is a remarkable variation in the disease manifestations and clinical course, suggesting that sarcoidosis reflects not a single disease entity but rather a collection of different granulomatous diseases. Overall, 70-80% of patients go into remission within the first two years [1], while other patients have unremitting multiorgan granulomatous inflammation sometimes associated with progressive fibrosis and organ failure. The frequency and different clinical manifestations of sarcoidosis varies between different geographic areas and ethnic groups [2]. Due to the partial clinical, radiological and pathological resemblance with tuberculosis, numerous studies on *Mycobacterium tuberculosis* have been published providing evidence for a role in sarcoidosis disease pathogenesis [3-6]. In one study, it was even estimated that 50% of sarcoidosis patients from the United States and Sweden have T memory lymphocytes for antigens of *M. tuberculosis* [7]. In a Japanese cohort, the QuantiFERON-TB test was only positive in 3.3% of patients [8], indicating that further studies in different parts of the world are required to clarify the potential involvement of *M. tuberculosis* in the pathogenesis of sarcoidosis.

The intracellular bacterium *Propionibacterium acnes* has also been extensively studied as a possible causative agent in sarcoidosis. Detection of DNA from propionibacteria in sarcoid lymph nodes, isolation of the bacteria from sarcoid lesions and studies in mice have suggested an etiological link [9-11]. In one study it was found that 35% of Japanese sarcoidosis patients had an in-vitro response to a specific antigen of *P. acnes*, while none of the healthy controls responded [12]. It is important to state the *P. acnes* is a human commensal found predominantly on the skin, but can also be isolated from the conjunctiva, external ear canal, mouth, upper respiratory tract and intestine[13].

Taken together, there is growing evidence for a role of both *M. tuberculosis* and *P. acnes* in the disease pathogenesis of sarcoidosis, which seems to be influenced by both geographic location and ethnicity. The aim of this study is to assess the T-cell response against both bacteria in Dutch sarcoidosis patients. Furthermore, we

investigated if our *P. acnes* based in vitro γ-interferon assay can be used to identify distinct clinical phenotypes within the population of sarcoidosis patients.

Material and Methods

Study subjects

Patient files from all 42 sarcoidosis patients treated with infliximab in our hospital between January 2005 and June 2010 were studied. Data regarding the screening for latent tuberculosis (LTBI) using an Interferon-Gamma Release Assay (TB-IGRA) was collected. These patients were selected based on the fact that a TB-IGRA is part of the pre-treatment protocol of infliximab. During treatment with infliximab, patients with a LTBI are at risk for developing active tuberculosis. Therefore, when sarcoidosis patients are diagnosed with LTBI they receive isoniazid (INH) for 6 months prior to their infliximab treatment.

Furthermore, other sarcoidosis patients, not eligible for infliximab, visiting the outpatient clinic were asked to donate blood for this study. A total of 40 patients was included. From this group, the patients without immunosuppressive drugs were used for the study (n=16). From the Dermatology outpatient clinic, 3 patients with acne vulgaris requiring isotretinoine (Roaccutane®) were also asked to participate. The control subjects comprised 11 healthy Dutch Caucasian employees of the St Antonius Hospital in the Netherlands. Demographic and clinical characteristics of the study subjects are summarized in table 1. By completing a questionnaire, relevant background information was provided by these volunteers and included medication, ethnicity and hereditary diseases. Verbal and written consent was obtained from all subjects. The study was approved by the Ethics Committee of the St. Antonius Hospital, Nieuwegein.

Cohort	Variables	Data
Sarcoidosis patients		
Infliximab	Total	42
	Male/Female	22/20
	Mean age (years)	50.3
	CXR stage(0/I/II/III/IV)	7/5/7/6/17
	Löfgren/non-Löfgren	0/42
	Immunosuppressive therapy yes/no	24/18
Outpatient	Total	16
	Male/Female	8/8
	Mean age (years)	50.6
	CXR stage(0/I/II/III/IV)	8/0/0/7/1
	Löfgren/non-Löfgren	3/13
	Immunosuppressive therapy yes/no	0/16
Acne patients	Total	3
	Male/Female	2/1
	Mean age (years)	23.3
	Immunosuppressive therapy yes/no	0/3
Healthy controls	Total	11
	Male/Female	5/6
	Mean age (years)	36.7

Table 1. Demographic and clinical characteristics of study subjects. CXR stage according to Scadding criteria: stage 0 = no lymphadenopathy, no parenchymal involvement; stage I = hilar lymphadenopathy only; stage II = hilar lymphadenopathy + parenchymal involvement; stage III = parenchymal involvement only; stage IV = pulmonary fibrosis present. Immune suppressive therapy indicates treatment with prednisone or methotrexate.

Interferon gamma release assays

From the 40 patients who were prospectively retrieved from the outpatient clinic and the 11 healthy controls, venous blood was drawn. Cord blood mononuclear cells (CBMC) were obtained from normal full-term vaginal deliveries under the auspices of the Department of Obstetrics and Gynaecology, University Medical Centre Utrecht, The Netherlands. Cord blood was collected using a heparinized 30ml syringe after delivery of the infant and ligation of the cord. Both CBMC and PBMC were isolated using Ficoll-Paque centrifugation and stored in liquid nitrogen before further use.
For the TB-IGRA both Tspot-TB® and QuantiFERON-TB Gold assays are used in our hospital. Both assays were performed following the manufacturer's instructions

(Tspot-TB®, Oxford Immunotec, United Kingdom, QuantiFERON-TB Gold, Cellestis, Australia). The antigens used in both IGRAs are Early Secretory Antigen Target –6 (ESAT6) and Culture Filtrate protein-10 (CFP10). PHA was used as a positive control to test general responsiveness of T-cells.
In the infliximab cohort, 40 patients had a Tspot-TB® performed and 2 patients a QuantiFERON-TB Gold. Patients from the outpatient cohort and healthy controls were all tested using the Tspot-TB®.
For the propionibacteria in vitro γ-interferon assay, a commercially available isolate of *P. acnes* (ATCC 6919) and a clinical isolate of *P. granulosum* were used ($35x10^6$/well). *P. granulosum* was identified based on morphology, gram staining and both indolase and catalase producing characteristics and 2 different clinical isolates were pooled. PBMCs were seeded on 96-well plates at a density of 100.000 per well in a final volume of 200µl. Phytohemagglutinin (0.5µg/ml, Murex) and medium were used as positive and negative control, respectively. The incubation period was 48 hours. Preliminary experiments with 24 hours incubation time yielded inconsistent results. The IFN-γ based propionibacteria ELISPOT was performed following manufacturer's instructions (IFN-γ T-SPOTTB Ready-Set-Go!, eBioscience). The number of spots were counted using Image J software (http://rsbweb.nih.gov/ij/index.html).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software). A Student's *t*-test was used to test the significance of differences in the number of spots between groups. Correlation analysis was performed using a Pearson test. A p value of < 0.05 was considered significant.

Results

In vitro γ-interferon response to *M. tuberculosis*

Table 2 summarizes the number of positive IGRAs in sarcoidosis patients and healthy controls. In the infliximab cohort, 2/42 (4.8%) of all patients had a positive IGRA as compared to 1/15 (6.7%) of the patients in the outpatient cohort. None of the tested healthy controls had a positive IGRA.

Cohort	Positive IGRA	% of cohort
Sarcoidosis patients		
Infliximab (n=42)	2	4.8
Outpatient (n=15)	1	6.7
Healthy controls (n=10)	0	0

Table 2. In vitro γ-interferon response to *M. tuberculosis*. The 2 positive IGRAs in the infliximab cohort were Tspot-TB®. The Tspot-TB® assay was positive when the difference between the number of spots counted after stimulation with ESAT6 or CFP10 minus the spots counted in the negative controls was 6 or more. In the outpatient group only 15/16 patients were tested due loss of material of the last patient. The negative result of one of the healthy controls was not included due to an insufficient response to PHA (16 spots, indeterminate result). IGRA = interferon-gamma release assay.

In vitro γ-interferon responses to *P. acnes* and *P. granulosum*

The results of the in vitro γ-interferon response to *P. acnes* and *P. granulosum* are presented in figure 1. All healthy controls as well as patients demonstrated a T-cell response to *P. acnes* and *P. granulosum*. No difference was found in the mean number of spots induced by either *P. acnes* or *P. granulosum* between healthy controls, CBMC and all patient groups. Although non-significant, differences were found in the response to PHA in the sense that healthy controls showed a higher response than sarcoidosis patients (p=0.06). Due to these differences in the response to the positive control PHA between healthy controls and sarcoidosis, the relative response to propionibacteria was also calculated by using the response to PHA as a marker for immunocompetence regarding IFN-γ production. The relative responses to propionibacteria are presented in figure 2.

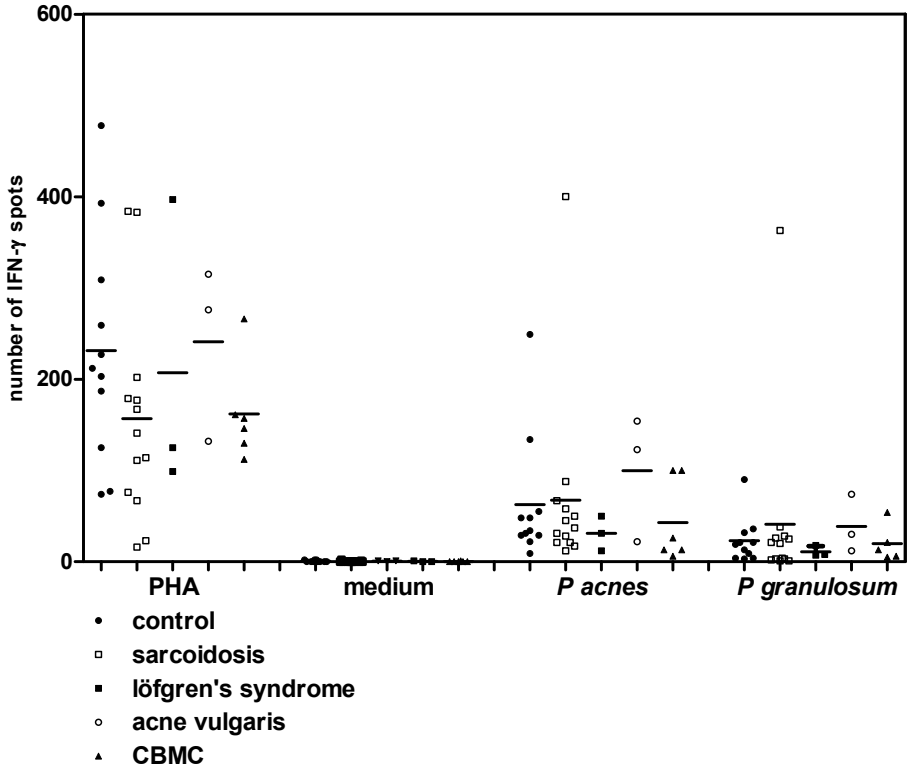


Figure 1. Number of IFN-γ spots per 100.000 mononuclear cells induced by different stimuli in healthy controls (n=11), sarcoidosis patients (n=13), patients with Löfgren's syndrome (n=3), patients with acne vulgaris (n=3) and cord blood mononuclear cells, CBMC (n=6). There is a non-significant difference between the number of spots induced by PHA between healthy controls and sarcoidosis patients (p=0.06). Mean values are shown by horizontal bars.

A small but non-significant higher relative response to *P.acnes* was found in sarcoidosis patients as compared to healthy controls. No differences were found in the *P. acnes* response between healthy controls, CBMC or other patient groups. Furthermore, within sarcoidosis patients, the data suggest two different populations; low responders and high responders. No differences were found in the relative response to *P. granulosum* between healthy controls, CMBC or patients.

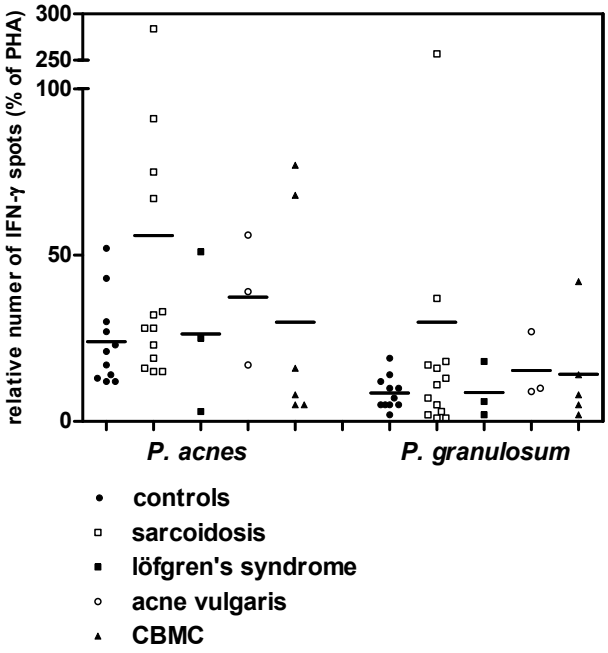


Figure 2. Number of IFN-γ spots induced by *P.acnes* or *P.granuslosum* as a percentage of the IFN-γ spots induced by PHA. Peripheral blood mononuclear cells of healthy controls (n=11), sarcoidosis patients (n=13), patients with Löfgren's syndrome (n=3), patients with acne vulgaris (n=3) or cord blood (n=6) were cultured for 48 hrs. Sarcoidosis patients show a slightly higher but non-significant relative amount of spots when stimulated with *P. acnes* as healthy controls (p=0.09). Mean values are shown by horizontal bars.

Correlation between T-cell responses to PHA and both *P. acnes* and *P. granulosum*

The data in figure 1 indicate that all healthy controls demonstrate an in vitro T-cell response to both *P. acnes* and *P. granulosum*. To test whether this response is based on antigen driven activation of memory T-cells or due to a polyclonal effect, mononuclear cells derived from cord blood were tested based on the assumption that the T-cells present are immunologically naïve. All CBMCs demonstrated a response to *P. acnes* and 5 of 6 CMBCs also responded to stimulation with *P. granulosum* (figure 1). Next, the responses to the mitogen PHA and both propionibacteria were compared. A strong correlation was found between the response to PHA and both propionibacteria (figure 3). The data therefore indicate that the IFN-γ spots induced by stimulation with propionibacteria are not due to antigen-specific activation but to a polyclonal effect.

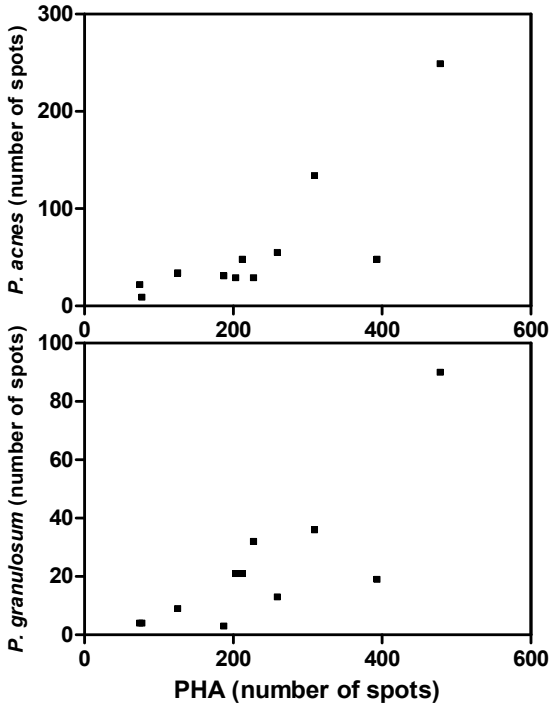


Figure 3. Correlation between IFN-γ spots per 100.000 mononuclear cells induced by PHA and *P.acnes* or PHA and *P.granulosum* in healthy controls. Correlation between PHA and *P.acnes*; r^2=0.63, p=0.004. Correlation between PHA and *P. granulosum*: r^2=0.64, p=0.003.

In vitro γ-interferon response to *P. acnes* in sarcoidosis patients

The data presented in figure 2 show the great variation in the in vitro γ-interferon response to *P. acnes* in sarcoidosis patients. The distribution even suggested the presence of 2 distinct groups, low responders and high responders. While the small sample size precludes statistical significant differences, an analysis was made of potential clinical differences between both groups. A remarkable finding was that 3 out of 4 high responders were sarcoidosis patients with extra pulmonary manifestations compared to 2 out of 9 low responders (figure 4).

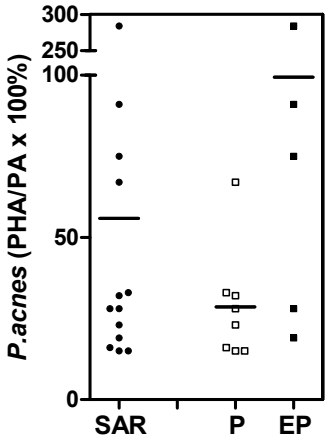


Figure 4. Relative number of IFN-γ spots induced by *P. acnes* as a percentage of the IFN-γ spots induced by PHA in sarcoidosis patients (SAR). Mean values are shown by horizontal bars. Separate analysis of patients with pulmonary (P) and extra pulmonary sarcoidosis (EP) revealed no significant differences (p=0.11).

Discussion

In the present study we have investigated the in vitro γ-interferon response against *Mycobacterium tuberculosis, Propionibacterium acnes* and *Propionibacterium granulosum* in Dutch sarcoidosis patients. A major issue in analyzing IFN-γ responses is the fact that there appears to be an intrinsic defect in IFN-γ producing capacity in peripheral blood mononuclear cells of sarcoidosis patients. In the present study we indeed found a decreased IFN-γ response to PHA in sarcoidosis patients as compared to healthy adults, in line with previous studies [14-16].

Regarding the IFN-γ response to mycobacterial antigens we used 2 different cohorts of sarcoidosis patients. In the cohort of patients receiving infliximab the prevalence of a positive IGRA was 4.8%. For this patient group no matched group of healthy controls was available. Comparison with data from the national database on prevalence of latent tuberculosis infection in the Netherlands was not considered useful based on the small number of patients in our infliximab cohort [17]. The prevalence of a positive IGRA in the outpatient cohort of sarcoidosis patient was 6.7% and comparable with the data from the infliximab cohort.

It is important to mention that one of the 2 patients with a positive IGRA in the infliximab cohort developed a digit infection with *Mycobacterium marinum* during treatment with infliximab. It is known that the Tspot-TB® can cross-react with non-tuberculous mycobacteria such *as M. kansasii, M. szulgai* and *M. marinum*. Therefore, it is possible that the positive Tspot-TB® result in this patient was due to cross-reactivity with *M. marinum* and thus could be considered false positive. On the other hand, non-tuberculous mycobacteria, also capable of producing virulence proteins [18], could also play a role in sarcoidosis. Overall, our data do not provide a strong link between sarcoidosis and previous infection with *M. tuberculosis* in The Netherlands. However, this does not totally exclude the possibility that *M.tuberculosis* causes sarcoidosis. The low prevalence of latent tuberculosis infection in Dutch sarcoidosis patients is in accordance with the (low) prevalence of tuberculosis in The Netherlands (5.8 cases per 100.000 inhabitants, data obtained from www.who.org). Interestingly, however, such a relationship was not found in Sweden or USA, both countries with an almost equal low prevalence of tuberculosis in the population (4.6 and 3.1 cases per 100.000 inhabitants respectively, www.who.org) but a much higher prevalence of latent tuberculosis in sarcoidosis patients [7]. In Japan the opposite is found: Inui *et al*. describes a low prevalence of latent tuberculosis infection in sarcoidosis patients in contrast to a higher prevalence of tuberculosis in Japan (28 cases per 100.000 inhabitants, www.who.org)[8]. The observed differences may be partially explained by the in vitro diagnostic techniques used. In the study with patients from Sweden and the USA, mycobacterial catalase-peroxidase (mKatG) was used as an antigen in the ELISPOT assay [7]. It has been suggested that mKatG is a dominant antigen within the T-cell repertoire of anti-mycobacterial responses in sarcoidosis patients. In both the Japanese [19] and present study ESAT-6 and CFP10 were used and not mKatG. However, in another cohort of sarcoidosis patients tested in the US, 30% had a T-cell response to ESAT-6, used also in the present study [20]. This indicates that there still is a striking difference in prevalence of latent tuberculosis infection in sarcoidosis patients between the US and The Netherlands. Comparison of the IFN-γ response to propionibacterial antigens between healthy controls and sarcoidosis patients was hampered by the difference in responses induced by PHA as mentioned earlier. Importantly, we found a correlation between

the IFN-γ response against PHA and *P. acnes* as well as *P. granulosum*, indicating possible mitogenic activity of both bacteria or the presence of superantigenic stimulation capacity. Both a mitogenic as well as an antigenic route for propionibacteria has already been suggested by Jappe and colleagues [21]. Also our data with cord blood mononuclear cells indicate that propionibacteria are mitogenic for human T lymphocytes. This is an important observation regarding the suspected etiological link between propionibacteria and sarcoidosis. The intriguing question remains how a commensal becomes a pathogen. In theory, based on mitogenic activity, *P.acnes* could act as an adjuvant and stimulate an ongoing immune response provoked by an unrelated antigen. Therefore, *P. acnes* does not necessarily has to be a causative agent in sarcoidosis disease pathogenesis. If *P. acnes* is involved in some patients with sarcoidosis, either based on an antigen or mitogenic mechanism, it is interesting to recognize this group and compare clinical characteristics and disease outcome with other sarcoidosis patients. The *P. acnes* IFN-γ spot assay with whole bacteria used in this study is not specific enough to address this question. This is also suggested by the fact that no differences were found between healthy controls and patients with acne vulgaris, a disease with an obvious role for *P. acnes*.

The immunopathology of sarcoidosis takes place in the lung compartment and it can be questioned whether the response of blood T lymphocytes is a relevant surrogate for the mucosal response. It therefore remains to be demonstrated whether the differences we found in blood will be present in the pulmonary compartment. Others have demonstrated that pulmonary T lymphocytes have a restricted TCR expression pattern, indicative for a oligoclonal expansion driven [22-24]. Furthermore, the responding T lymphocytes in the lungs of sarcoidosis patients are CD27 negative while in blood these T lymphocytes are CD27 positive [25]. This suggests that the cellular immune response in the lung is an ongoing process.

Characterization of sarcoidosis patients based on the presence or absence of *P. acnes* in the lungs as detected by PCR in combination with a more sophisticated and antigenic driven *P. acnes* ELISPOT in both peripheral blood and BAL fluid should be the next steps in further research. The involvement of *P. acnes* in sarcoidosis can also be substantiated by analysis of antibody patterns against cell lysates of *P.acnes*

as has been performed in acne patients [26]. If one could link propionibacteria to a distinct group of sarcoidosis patients, randomized controlled trials using antibiotics or even heat-inactivated *P.acnes* vaccines [27] could open new therapeutic options.
In summary, our data do not provide a strong link between sarcoidosis and previous infection with *M. tuberculosis* in The Netherlands. Comparison of the in vitro responses to propionibacteria was hampered by the observations that sarcoidosis patients have an overall decreased IFN-γ producing capacity in blood and the suggested mitogenic capacity of these bacteria. Therefore, to determine if there is a subgroup of patients with propionibacteria as source of persisting antigen, a sarcoidosis specific antigen of these bacteria should be identified which subsequently can be used in an in vitro γ-interferon assay.

Reference List

1. Grunewald, J. & Eklund, A. Sex-specific manifestations of Lofgren's syndrome. *Am. J. Respir. Crit Care Med.* **175**, 40-44 (2007).
2. Rybicki, B. A. & Iannuzzi, M. C. Epidemiology of sarcoidosis: recent advances and future prospects. *Semin. Respir. Crit Care Med.* **28**, 22-35 (2007).
3. Gupta, D., Agarwal, R., Aggarwal, A. N. & Jindal, S. K. Molecular evidence for the role of mycobacteria in sarcoidosis: a meta-analysis. *Eur. Respir. J.* **30**, 508-516 (2007).
4. Oswald-Richter, K. *et al.* Mycobacterial ESAT-6 and katG are recognized by sarcoidosis CD4+ T cells when presented by the American sarcoidosis susceptibility allele, DRB1*1101. *J. Clin. Immunol.* **30**, 157-166 (2010).
5. Oswald-Richter, K. A. *et al.* Cellular responses to mycobacterial antigens are present in bronchoalveolar lavage fluid used in the diagnosis of sarcoidosis. *Infect. Immun.* **77**, 3740-3748 (2009).
6. Song, Z. *et al.* Mycobacterial catalase-peroxidase is a tissue antigen and target of the adaptive immune response in systemic sarcoidosis. *J. Exp. Med.* **201**, 755-767 (2005).
7. Chen, E. S. *et al.* T cell responses to mycobacterial catalase-peroxidase profile a pathogenic antigen in systemic sarcoidosis. *J. Immunol.* **181**, 8784-8796 (2008).
8. Inui, N., Suda, T. & Chida, K. Use of the QuantiFERON-TB Gold test in Japanese patients with sarcoidosis. *Respir. Med.* **102**, 313-315 (2008).
9. Abe, C., Iwai, K., Mikami, R. & Hosoda, Y. Frequent isolation of Propionibacterium acnes from sarcoidosis lymph nodes. *Zentralbl. Bakteriol. Mikrobiol. Hyg. A* **256**, 541-547 (1984).
10. Eishi, Y. *et al.* Quantitative analysis of mycobacterial and propionibacterial DNA in lymph nodes of Japanese and European patients with sarcoidosis. *J. Clin. Microbiol.* **40**, 198-204 (2002).
11. McCaskill, J. G. *et al.* Pulmonary immune responses to Propionibacterium acnes in C57BL/6 and BALB/c mice. *Am. J. Respir. Cell Mol. Biol.* **35**, 347-356 (2006).
12. Ebe, Y. *et al.* Proliferative response of peripheral blood mononuclear cells and levels of antibody to recombinant protein from Propionibacterium acnes DNA expression library in Japanese patients with sarcoidosis. *Sarcoidosis. Vasc. Diffuse. Lung Dis.* **17**, 256-265 (2000).
13. Funke, G., von, G. A., Clarridge, J. E., III & Bernard, K. A. Clinical microbiology of coryneform bacteria. *Clin. Microbiol. Rev.* **10**, 125-159 (1997).
14. Bertran, G., Arzt, E., Resnik, E., Mosca, C. & Nahmod, V. Inhibition of interferon gamma production by peripheral blood mononuclear leukocytes of patients with sarcoidosis. Pathogenic implications. *Chest* **101**, 996-999 (1992).
15. Rottoli, P., Muscettola, M., Grasso, G., Perari, M. G. & Vagliasindi, M. Imparied interferon-gamma production by peripheral blood mononuclear cells and effects of calcitriol in pulmonary sarcoidosis. *Sarcoidosis.* **10**, 108-114 (1993).

16. Veltkamp, M. *et al.* Toll-like receptor (TLR)-9 genetics and function in sarcoidosis. *Clin. Exp. Immunol.* (2010).

17. Erkens C.G.M..Kalisvaart N.A & Slump E. Tuberculose in Nederland 2003 en 2004. Surveillance rapport over de tuberculosesituatie in Nederland. *Dutch ISBN-number: 90-77865-02-0* (2006).

18. van Ingen, J., de Zwaan,. R., Dekhuijzen, R., Boeree, M. & van, S. D. Region of difference 1 in nontuberculous Mycobacterium species adds a phylogenetic and taxonomical character. *J. Bacteriol.* **191**, 5865-5867 (2009).

19. Jugeau, S. *et al.* Induction of toll-like receptors by Propionibacterium acnes. *Br. J. Dermatol.* **153**, 1105-1113 (2005).

20. Drake, W. P. *et al.* Cellular recognition of Mycobacterium tuberculosis ESAT-6 and KatG peptides in systemic sarcoidosis. *Infect. Immun.* **75**, 527-530 (2007).

21. Jappe, U., Ingham, E., Henwood, J. & Holland, K. T. Propionibacterium acnes and inflammation in acne; P. acnes has T-cell mitogenic activity. *Br. J. Dermatol.* **146**, 202-209 (2002).

22. Grunewald, J. *et al.* Restricted V alpha 2.3 gene usage by CD4+ T lymphocytes in bronchoalveolar lavage fluid from sarcoidosis patients correlates with HLA-DR3. *Eur. J. Immunol.* **22**, 129-135 (1992).

23. Grunewald, J., Berlin, M., Olerup, O. & Eklund, A. Lung T-helper cells expressing T-cell receptor AV2S3 associate with clinical features of pulmonary sarcoidosis. *Am. J. Respir. Crit Care Med* **161**, 814-818 (2000).

24. Moller, D. R. Involvement of T cells and alterations in T cell receptors in sarcoidosis. *Semin. Respir. Infect.* **13**, 174-183 (1998).

25. Katchar, K., Wahlstrom, J., Eklund, A. & Grunewald, J. Highly activated T-cell receptor AV2S3(+) CD4(+) lung T-cell expansions in pulmonary sarcoidosis. *Am. J. Respir. Crit Care Med* **163**, 1540-1545 (2001).

26. Basal, E., Jain, A. & Kaushal, G. P. Antibody response to crude cell lysate of propionibacterium acnes and induction of pro-inflammatory cytokines in patients with acne and normal healthy subjects. *J. Microbiol.* **42**, 117-125 (2004).

27. Nakatsuji, T., Rasochova, L. & Huang, C. M. Vaccine therapy for P. acnes-associated diseases. *Infect. Disord. Drug Targets.* **8**, 160-165 (2008).

8

Summary and general discussion

This thesis addresses the question whether genetic or functional defects in Toll-like receptors involved in the innate immune recognition of bacteria play a role in disease susceptibility of sarcoidosis, or will influence the disease course. The studies described were motivated by the increasing recognition that innate immune responses can influence ensuing adaptive immune responses. Figure 1 summarizes the genes studied and shows locations of the single nucleotide polymorphisms (SNPs).

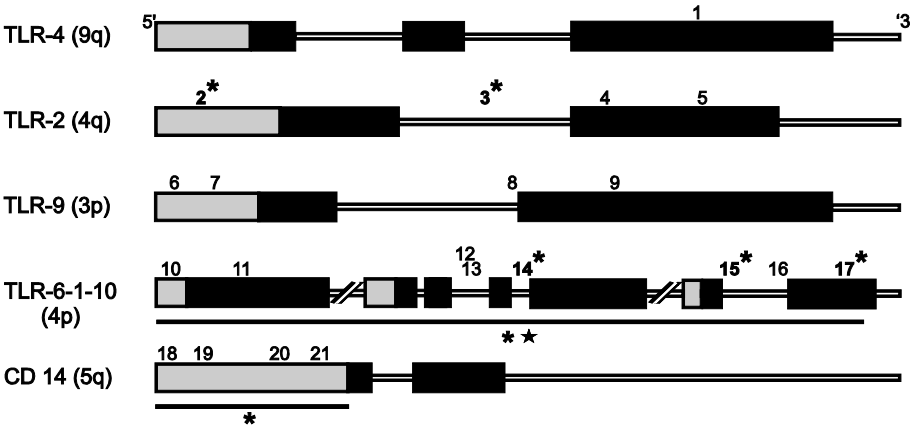


Figure 1. Summary of all genes studied in this thesis with numbered relative positions of the SNPs. Grey bar = promoter region, parallel horizontal lines = intron, black bar = exon. SNPs associated with sarcoidosis are highlighted in bold with an asterisk. Haplotypes associated with sarcoidosis are shown as black lines below the gene also marked with an asterisk. The haplotype associated with Löfgren's syndrome is shown as a black line below the gene marked with a star. Chromosomal positions in parentheses.

1 = rs4986790	2 = rs4696480	3 = intron 1 $(GT)_n$	4 = rs5743704
5 = rs5743708	6 = rs187084	7 = rs5743839	8 = rs352139
9 = rs352140	10 = rs5743794	11 = rs3775073	12 = rs5743594
13 = rs5743595	14 = rs5743604	15 = rs7658893	16 = rs4274855
17 = rs11096955	18 = rs5744441	19 = rs2569192	20 = rs5744455
21 = rs2569190			

Furthermore, T-cell responses against the intracellular bacteria *Mycobacterium tuberculosis*, *Propionibacterium acnes* and *Propionibacterium granulosum* were analyzed in order to investigate whether this could be causative agents in Dutch sarcoidosis patients or reveal new clinical phenotypes within the heterogeneous group of sarcoidosis patients.

Summary

Chapter 2 describes the genetic variation of the important TLR-4 single nucleotide polymorphism rs4986790(Asp299Gly) in sarcoidosis patients. TLR-4 is the first described TLR in humans and is capable of recognizing LPS, unique to the cell wall of Gram-negative bacteria. TLR-4 has also been shown to be involved in the recognition of endogenous ligands such as heat shock proteins (HSP60 and HSP70)[1] and cross-reactivity with mycobacterial heat shock proteins is suggested as a disease mechanism in sarcoidosis [2]. An A to G substitution of this polymorphism leads to the replacement of Aspartic acid with Glycine at amino acid 299, located in the extracellular domain of TLR-4. This substitution has been associated with LPS hypo responsiveness in humans [3]. No differences were found in allelic distribution of the Asp299Gly polymorphism between sarcoidosis patients and healthy controls, nor between different clinical subsets of disease severity. These results are in line with the current concept of the role of bacteria in the pathogenesis of sarcoidosis. The two most plausible bacterial causative agents are the intracellular *M. tuberculosis* and the intracellular and Gram-positive *P. acnes*, both mainly recognized by TLR2 and not TLR-4 [4-6]. In a German population of sarcoidosis patients, an association with Asp299Gly was reported [7]. The frequency of the Asp299Gly polymorphism in the patients was similar to that in our sarcoidosis population. A major difference was however found in the frequency of this polymorphism in the control population, which is the most likely explanation for this discrepancy.

Chapter 3 describes the genetic variation and functional consequences of 3 SNPs and one tandem repeat polymorphism (GT_n) in the gene encodingTLR-2 in sarcoidosis patients and healthy controls. TLR-2 recognizes peptidoglycan from Gram-positive bacteria, mycobacterial proteins as well as endogenous proteins like HSP [8]. As mentioned earlier, TLR-2 is involved in the recognition of both *M. tuberculosis* and *P. acnes*. We found a higher prevalence of the AA genotype of promoter polymorphism -16934A/T in sarcoidosis patients with a chronic disease course as compared to patients with a self-remitting form of the disease. This association however could not be replicated in a validation cohort of Dutch sarcoidosis patients

suggesting that genetic variation in TLR2 is not a major disease susceptibility factor in sarcoidosis. Interestingly, we furthermore found a highly significant association between this TLR2 promoter polymorphism and the number of GT-tandem repeats in the intron 1 polymorphism. The mean number of GT tandem repeats was the lowest in the AA-genotype group and the highest in the TT-genotype group. The functional experiments that followed this observation demonstrated an inverse correlation between the production of TNF-α, IL-12 as well as IL-6 and the number of GT-repeats after stimulation with TLR-2 agonists. The fact that subjects carrying the -16934 AA genotype, which is suggested to have a higher prevalence in patients with chronic sarcoidosis, produced significantly more TNF-α upon stimulation with TLR-2 agonists fits in the current model of sarcoidosis pathogenesis. TNF-α is important in the initiation and perpetuation of inflammation in sarcoidosis, contributing to progressive disease with or without development of fibrosis. Various clinical trials demonstrated that blocking TNF-α using antagonists such as infliximab or etanercept [9] results in clinical improvement and lowers the requirement of corticosteroids. Based on our results it is tempting to speculate that patients with the TLR2 -16934 AA-genotype will produce higher amounts of TNF-α during continuous innate immunity stimulation by a persisting antigen compared to patients with either the AT- or TT-genotype, thereby increasing their risk of developing chronic disease.

Chapter 4 presents the genotyping and haplotype formation of 4 SNPs in the promoter region of the gene encoding for CD14. CD14 interacts with various pathogen-associated molecular patterns and is capable of forming a receptor complex with TLR-2 [10]. Two of these SNPs are suggested to increase CD14 expression in vitro [11,12]. A significant higher prevalence of a low frequency promoter haplotype was found in sarcoidosis patients with a chronic disease course compared to healthy controls. Based on the low frequency of this haplotype in the control population, genetic variation in the CD14 promoter region seems not a major disease susceptibility locus in sarcoidosis. However, the observation that this CD14 haplotype is more prevalent in chronic sarcoidosis patients in interesting in light of the suggested higher prevalence of the TLR2 -16934AA genotype in patients with chronic disease as well.

Chapter 5 evaluates possible genetic or functional differences in TLR-9 between sarcoidosis patients and healthy controls. The endosomal localized receptor TLR-9, capable of recognizing unmethylated nucleic acid motifs, is one of the most important receptors in the initiation of protective immunity against intracellular pathogens and necessary for an adequate immune response against both *M. tuberculosis* and *P. acnes* [13,14]. A total of 4 SNPs, located in the promoter region, intron 1 and exon 2 of the gene encoding TLR-9, were genotyped. We did not find an association between these polymorphisms and disease susceptibility or influence on disease course in Dutch sarcoidosis patients. To rule out genetic defects other than the known polymorphisms, the promoter region as well as exon 1 and 2 were sequenced in 20 patients revealing no new mutations. In order to fully explore the role of TLR-9 in sarcoidosis, IL-6, IFN-γ and IL-23 production capacity upon stimulation with a TLR-9 agonist was investigated in a small group of patients and controls. Sarcoidosis patients demonstrated a decreased IFN-γ production capacity upon stimulation with different stimuli, suggestive for the anergic state of peripheral T-cells often seen in sarcoidosis patients [15-18]. Interestingly, the data also suggested a reduced IL-23 production capacity after stimulation with a TLR-9 agonist in sarcoidosis. IL-23 is an important cytokine in the recently discovered Th-17 pathway, which can be induced by many pathogens including *P. acnes* and *M. tuberculosis* [19]. Further genetic as well as functional studies are needed to determine if the Th-17 pathway is important in sarcoidosis pathogenesis.

In **chapter 6**, data is presented regarding genetic variability of 8 SNPs located in the TLR10-TLR1-TLR6 gene cluster. As mentioned earlier, TLR-2 senses a variety of microbial components and it has been shown that TLR-1 and TLR-6 are co-receptors for TLR-2, forming either TLR-2/1 or TLR-2/6 heterodimers with different agonist specificities [20]. Recently, it has been suggested that TLR-10 also functions as a co-receptor for TLR-2 [21]. Three of these SNPs, two in TLR10 and one in TLR1, had a decrease of major allele frequency in both sarcoidosis patients as well as patients with Löfgren's syndrome, although only statistical significant in the first group. Separate analysis of sarcoidosis patients with self-remitting or chronic disease course revealed that the differences found for the three SNPs are due to a low

prevalence of the major alleles in chronic sarcoidosis. Nucleotide substitution at rs11096955 (TLR10) leads to the replacement of Isoleucine by Leucine in the extracellular domain of the TLR-10 receptor, indicating the possibility of altered ligand specificity. Construction of haplotypes using all 8 SNPs revealed that the most common haplotype found was significantly decreased in patients with chronic sarcoidosis. In patients with Löfgren's syndrome, 2 haplotypes had a significantly higher prevalence compared to healthy controls. Interestingly, the prevalence of one of these haplotypes was also increased in sarcoidosis patients with a self remitting form of the disease indicating that it could also be a disease modifying haplotype. The result from this study suggests that absence of a common haplotype in the TLR10-TLR1-TLR6 gene cluster increases the risk of developing chronic disease in patients already affected by sarcoidosis. Two other haplotypes in the TLR10-TLR1-TLR6 gene cluster could predispose for developing Löfgren's syndrome or influence disease course. Interestingly, once again genetic differences in genes encoding for TLR-2 co-receptors were found in patients with chronic sarcoidosis.

Chapters 2-6 describe the experiments performed on the genetic polymorphisms in genes encoding for TLRs important for the innate recognition of bacteria. As mentioned earlier, the most studied possible causative bacterial agents in sarcoidosis pathogenesis are *M. tuberculosis* [22-25] and *P. acnes* [26-28]. In one study, it was estimated that 50% of sarcoidosis patients from the United States (US) and Sweden have T-cell memory for antigens of *M. tuberculosis* [29]. Another study demonstrated that 35% of a small cohort of Japanese sarcoidosis patients had an in-vitro response to a specific antigen of *P. acnes*, while none of the healthy controls responded [30]. In **chapter 7** we therefore evaluated T-cell responses against these bacteria in Dutch sarcoidosis patients. Using the Tspot-TB® assay, latent tuberculosis infection was detected in 5.3% of the 57 sarcoidosis patients tested. This is in concordance with a low prevalence of tuberculosis in The Netherlands but does not provide a strong link between sarcoidosis and previous infection with *M. tuberculosis* as in patients from the US or Sweden. Interpretation of the in vitro T-cell responses against *P. acnes* and *P. granulosum* was difficult based on a decreased IFN-γ production capacity we found in peripheral blood mononuclear cells of sarcoidosis, in line with the results

described in chapter 5. Based on these differences the relative responses against propionibacteria using the response to PHA as a marker for immunocompetence regarding IFN-γ production was compared, as described previously [31]. A small, non-significant difference between the relative response to *P.acnes* in sarcoidosis patients compared to healthy controls was found. No new clinical phenotypes were seen based on this *P.acnes* based IGRA. An important observation was that all healthy controls as well as immunologically naïve mononuclear cells derived from cord blood demonstrated a T-cell response to both *P. acnes* and *P. granulosum*. Indeed, a correlation between the IFN-γ response against PHA and *P. acnes* as well as *P. granulosum* was found, indicating possible mitogenic activity of both bacteria. This finding can provide new insights on how these commensal bacteria can play a role in sarcoidosis disease pathogenesis. Overall, the in vitro T-cell responses in peripheral blood to mycobacteria as well as propionibacteria do not allow to conclude on a major role for these microorganisms in the etiology and pathogenesis of sarcoidosis.

General discussion

Sarcoidosis is a granulomatous disease of unknown etiology with a great diversity in clinical manifestations. The current understanding of its pathogenesis is that several sequential immunological events are involved that finally result in granuloma formation: (1) exposure to one or several still elusive antigen(s), (2) activation of macrophages, (3) acquiring T cell immunity against the antigen(s) mediated by antigen processing and presentation by macrophages, (4) generation of specific T-effector cells and (5), induction of granuloma formation. These events take place on the background of a spectrum of genetic polymorphisms that influence disease susceptibility or outcome [32]. It is important to state that during an effective T cell response the formation of granuloma is not necessary. Interesting question is whether granuloma formation in sarcoidosis occurs because of the nature of the antigen/ micro organism or due to an ineffective T cell response.
This thesis focuses on the first two immunological events mentioned above. After exposure to a microorganism the immune system has to differentiate between self and non-self and, perhaps even more important, between danger and no-danger. The innate immune system is important in the first recognition of pathogens, mainly via recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) such as Toll-like receptors. All PRRs contain structures that link them to signaling cascades to initiate cell activation and subsequent release of cytokines, reactive oxygen intermediates or nitrogen oxide. It is important to mention that innate immune responses dictate the magnitude and direction of the subsequent adaptive immune responses.

In the case of chronic granulomatous disease and juvenile sarcoidosis (early-onset sarcoidosis), granuloma formation has been linked to specific defects in innate immunity [33,34]. For other granulomatous disorders such as tuberculosis, leprosy and Crohn's disease, defects in innate immune responses have also been suggested to play a role in disease pathogenesis [35-41]. It is therefore tempting to speculate that innate immunity could also be involved in sarcoidosis disease pathogenesis.

A total of 7 different innate immunity genes comprising of CD14 and 6 TLRs were analyzed in Dutch sarcoidosis patients. We have not analyzed genetic variation in components of the signaling cascade such as MyD88, IRAK4 and other candidates. Genetic variation in one or more SNPs localized in 4 genes were linked to sarcoidosis. Separate analysis of sarcoidosis patients with self-remitting or chronic disease course revealed that most of the genetic differences found were present in patients with a chronic form of sarcoidosis, indicative of a disease modifying effect of these genes. Interestingly, the 4 genes associated with sarcoidosis are all involved in TLR-2 expression and/or function. A genetic association was found for TLR-2 itself as well as for 3 different co-receptors of TLR-2 namely TLR-1, TLR-10 and CD14. These data are in agreement with the growing body of evidence that TLR-2 could play a role in sarcoidosis disease pathogenesis. Wikén and co-workers demonstrated a higher TLR-2 and TLR-4 expression in peripheral blood mononuclear cells of sarcoidosis patients compared to healthy controls [42]. Furthermore, an increased production of TNF-α was seen in patients after simultaneous stimulation with TLR-2 and NOD-2 ligands compared to healthy controls. Oswald-Richter and colleques showed TLR-2 dependent T-cell specific responses to mycobacterial antigens in BAL fluid of active sarcoidosis patients [43]. Chen *et al* [44] found an increased expression of Serum amyloid A (SAA) in sarcoid granulomas. SAA is an acute-phase reactant and one of the most highly expressed proteins induced during mycobacterial infection [45,46]. SAA activates NF-κβ and induces cytokine production from sarcoidosis alveolar macrophages via TLR-2. It regulates granulomatous inflammation through its effect on production of IFN-γ, TNFα and IL-10. They propose a model where SAA promotes chronic granulomatous inflammation by aggregating within granulomas providing a persistent stimulus as a ligand for TLR-2 and other innate receptors, within a matrix that promotes binding of relevant pathogenic antigens that stimulate local Th-1 responses (figure 2). The results of chapter 3 presented in this thesis could fit in this model. It was speculated that patients with the TLR2 -16934 AA-genotype have a higher risk of developing chronic sarcoidosis due to higher TNF-α production during ongoing innate immunity stimulation by a persisting antigen. This could be one of the probably many risk factors defining why some patients have a self-remitting disease and others develop chronic disease. The genetic variability in TLR1, TLR10 and CD14 found in patients with chronic disease could also influence TLR-2 function

in a negative way increasing the risk of chronic disease. An important limitation of this model is the fact that mycobacterial infection is postulated as a major risk factor for sarcoidosis. If mycobacterial infection is important in disease pathogenesis, it remains to be seen in which distinct subgroup of sarcoidosis patients this is the case. This prompted us to perform the research presented in chapter 7.

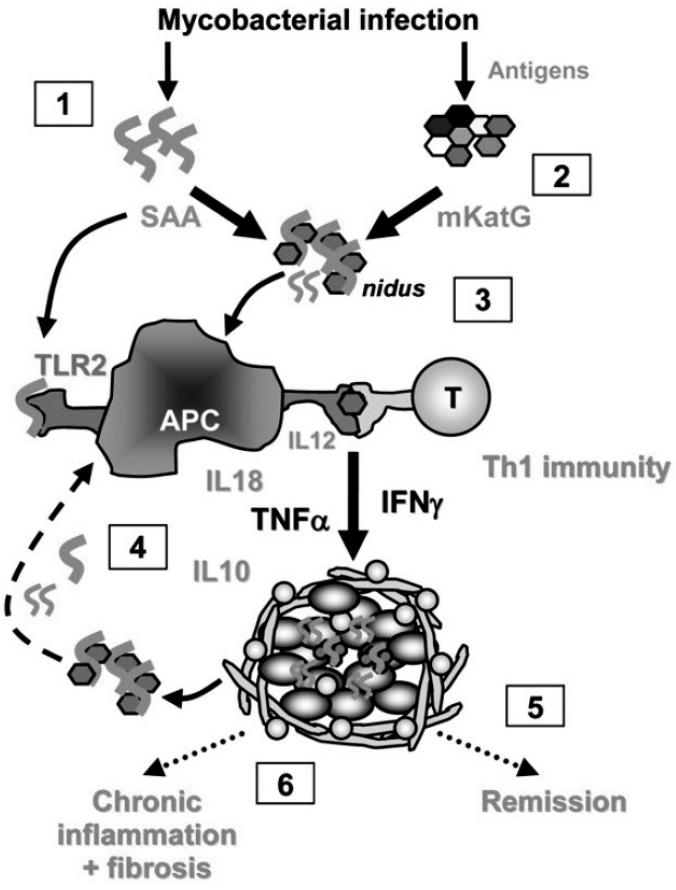


Figure 2. Model proposed by Chen *et al.*[44]. Serum amyloid A (SAA) as a pathobiologic bridge between mycobacterial infection and chronic granulomatous inflammation in sarcoidosis. In this model, SAA is locally induced within antigen-presenting cells in a granuloma by mycobacterial organisms, and up-regulated by an acute-phase response (1) that is part of an effective killing response that leaves remnants of mycobacterial proteins such as mKatG (2). SAA serves as a "seed" for further amyloid-like protein aggregation and binds to other matrix protein partners to form a nidus for epitheloid granuloma formation (3). SAA and its matrix partners function as a trap for microbial or auto-antigens within granulomas while

soluble SAA, released from tissue granulomas, serves as a ligand for TLR-2 to regulate Th-1 driven epitheloid granulomatous inflammation through various cytokines such as TNF-α, IL-18 and IL-10 (4). Granulomatous inflammation resolves only after clearance of SAA and local pathogenic antigens (5). In unremitting sarcoidosis, ineffective degradation and clearance of SAA and pathogenic antigens leads to chronic inflammation and/or fibrosis (6). Publication with permission of the journal.

In order to investigate the contribution of mycobacteria in the etiopathogenesis of sarcoidosis, Interferon Gamma Release Assays (IGRA) on peripheral blood mononuclear cells was performed in a cohort of Dutch sarcoidosis patients. Latent tuberculosis, defined as detection of a T-cell memory response against antigens of *M. tuberculosis* and indicative of presence of *M. tuberculosis* not revealing themselves through any clinical or other signs of active disease in the host, was found in 3 of 57 patients tested (5.3%). This percentage is in concordance with the low prevalence of tuberculosis in The Netherlands but, therefore, does not provide a strong link with previous *M. tuberculosis* infection and sarcoidosis in Dutch patients. It is important to mention that mKatG, the mycobacterial antigen used in determining latent tuberculosis infection in the US and Sweden [29] was not used in the IGRA performed in our study. However, 30% of US sarcoidosis patients showed a positive T-cell response to ESAT-6, another mycobacterial antigen which is included in the IGRA used in our experiments [47]. One could speculate that some nontuberculous mycobacteria ,also capable of producing virulence proteins [48], can play a role in sarcoidosis disease pathogenesis. This was suggested by the fact that one of the Dutch sarcoidosis patients receiving infliximab described in chapter 7 developed a digit infection with *Mycobacterium marinum*. Infection with nontuberculous mycobacteria can result in formation of noncaseating granulomas and exposure in the environment is abundant [49]. Although most pulmonary infections with nontuberculous mycobacteria result in cavitations and bronchiectasis, diffuse interstitial patterns have been described [50]. The next step in elucidating a possible role for mycobacteria in Dutch sarcoidosis patients should be to perform an IGRA using mKatG in order to better compare these results with previous studies.

When mycobacteria thusfar may be involved in only 5% of sarcoidosis patients, there is room for postulating other microbial causative agents in sarcoidosis. A possible

role for *P. acnes* and *P. granulosum* was therefore studied by in vitro activation of peripheral blood monocytes with *P. acnes* or *P. granulosum*. A major problem in the interpretation of these experiments was the fact that T-cells in peripheral blood of sarcoidosis patients have a decreased IFN-γ producing capacity, also when stimulated with polyclonal activators such as PHA, a finding in concordance with previous studies [15,18,51]. This anergic state could be part of the immunological paradox stating that sarcoidosis is characterized by an excessive Th-1 response in the affected organs whereas circulating T-cells poorly respond to antigen challenge. It could be that peripheral T-cells are less responsive to the causative agents based on homing of activated and well-responsive T-cells to the site of disease activity such as the pulmonary compartment. It has also been suggested that the population of regulatory T cells is amplified in circulating blood as well as BAL fluid of patients presenting with active sarcoidosis [52,53]. The presence of a small number of antigen reactive T cells or memory T cells in the peripheral blood compared to the pulmonary compartment could lead to a regulatory T cell/memory T cell disequilibrium as a possible mechanism of anergy. However, a decreased IFN-γ production can be seen in patients who are already in remission of sarcoidosis indicating a more structural defect in IFN-γ producing capacitiy of T-cells.

The decreased IFN-γ producing capacity of sarcoidosis found in peripheral blood mononuclear cells hampered comparison between healthy controls and patients using an IFN-γ based assay. This indicates that such an IGRA is not likely to reveal a distinct subgroup of patients with propionibacteria as causative agents. Future studies should seek for possible sarcoidosis specific antigens of propionibacteria. In a Japanese study it was found that 35% of a small cohort of sarcoidosis patients had an in-vitro response to a specific antigen of *P. acnes*, while none of the healthy controls responded [30]. This antigen, RP35, was recovered from a λgt11 genomic DNA library of *P. acnes* by immunoscreening with sera from patients with sarcoidosis. Experimentally induced hypersensitivity to RP35 led to pulmonary granulomas in 30% of the tested mice [54]. In other experimental models of allergic diseases such as encephalomyelitis, thyroiditis and orchitis using self-antigens it was demonstrated that auto-immune inflammatory lesions are induced in the organs from which the

self-antigens originated [55-57]. This could indicate that the RP35 antigen induced pulmonary granulomas only in mice in which *P. acnes* normally resides in the lungs. Although not a causative explanation, *P. acnes* could indeed be cultured from 33% of normal healthy mice. In human studies, *P. acnes* could not be cultured from BAL fluid in patients nor healthy controls [58]. *P. acnes* DNA was detected in 70% of the patients compared to 21% in other lung diseases. Interestingly, in situ signals of *P. acnes* DNA were detected in the cytoplasm up to 2.8% of alveolar macrophages from sarcoidosis patients, but not from cells of control patients. Although Koch's postulates are not met, the data at least suggest a role for *P. acnes* in sarcoidosis in Japanese patients. The data presented in chapter 7 of this thesis could provide new insights on how these commensal bacteria can play a role in sarcoidosis disease pathogenesis. The suggested mitogenic activity could indicate that *P.acnes* can act as an adjuvant and stimulate an ongoing immune response provoked by an unrelated antigen. Immunomodulation by *P.acnes* has been previously described [59]. This could implicate that *P.acnes* does not necessarily has to be a causative agent in sarcoidosis.

Up to the 1970s the diagnosis of sarcoidosis was also based on the *in vivo* reaction to the so-called Kveim antigen in the Kveim-Siltzbach test[60]. This preparation consisted of a homogenate of spleen or lymp nodes of sarcoidosis patients. In patients with sarcoidosis, approximately 4 weeks after intracutaneous injection of Kveim antigen, non-caseating granulomas identical with sarcoid granulomas could be found at the injection site. We have obtained this material and used it for in vitro stimulation of peripheral blood T lymphocytes of sarcoidosis patients and controls. Although in vitro culture of PBMCs obtained from sarcoidosis patients for 48 hrs with relative high doses of Kveim antigen (1:100 final dilution) could result in the induction of γ-interferon spot forming cells, the results were inconsistent. This indicates that even when a known sarcoidosis inducing antigen is used, technical aspects of an IGRA could influence the results. Still, the Kveim reagent could be the haystack containing the golden needle and deserves further testing.

Müller- Quernheim *et al.* did not find any bacterial DNA when analyzing Kveim reagent in the previous century[61]. Based on the growing amount of data that mycobacterial antigen mKatG could be a causative agent in sarcoidois it would be interesting to develop a mKatG specific PCR and test the available Kveim reagent we have obtained.

Future directions

As described in this thesis, TLR-2 and co-receptors seem important in some patients with sarcoidosis. An unanswered question is in which distinct population of the heterogeneous group of sarcoidosis patients they are. Searching for T-cell memory responses against bacteria recognized by TLR-2 in Dutch sarcoidosis patients did not reveal this suggested distinct new clinical phenotype of patients. Maybe the role of bacteria in sarcoidosis is rather small and research should focus more on an endogenous antigen for this disease, for instance using techniques as proteomics [62].
It is known that TLRs are also capable of recognizing endogenous ligands. Because these ligands are released in association with tissue injury the term damage-associated molecular patterns (DAMPs) is suggested. DAMPs comprise of molecules such as heat-shock proteins (HSPs), high mobility group box-1 (HMGB1), fibronectin, collagen, hyaluronan, calcium binding proteins or defensins [63]. A relationship between HSPs and sarcoidosis was already suggested by Dubaniewicz et al. [2]. In light of recognizing endogenous ligands, other TLRs not studied in this thesis should be investigated with a special interest in TLR-3. Defective TLR-3 function has been demonstrated to predispose for progressive pulmonary fibrosis in sarcoidosis (C. Donnelly *et al*, abstract 3430 presented at the ERS Barcelona 2010). An increased frequency of the TLR3 SNP Leu412PHe was found in both Irish and American patients with sarcoidosis and demonstrated a decreased IFN-β induction after fibroblast stimulation with Poly I:C.
Future research in elucidating possible causative agents for sarcoidosis is necessary in order to define new clinical phenotypes in sarcoidosis. Dividing patients by chest X-ray according to the Scadding criteria [64] has limitations in defining clinical phenotypes of sarcoidosis. HRCT scoring of patients with sarcoidosis will enable a

more detailed characterization of phenotypical differences and seems to be more sensitive in predicting respiratory disability [65]. Interesting future experiments will be to set up an interferon-gamma release assay (IGRA) using the most possible causative agents for sarcoidosis as antigenic stimuli (table 1, general introduction chapter). In theory, if micro organisms such as *P. acnes* can be linked to a distinct group of sarcoidosis patients it could lead the way to randomized controlled trials using antibiotics to see whether decreasing the bacterial load in the lungs is beneficial for these patients.

Another interesting technique worth mentioning in light of the search for new disease phenotypes is Fibred Confocal Fluorescence Microscopy (FCFM) which can provide microscopic imaging of living tissue. For example, using a confocal fluorescence bronchoalveoscopy, the presence of lipoproteinaceous material in the alveoli and alveolar macrophages was demonstrated in vivo in a patient with pulmonary alveolar proteinosis (PAP) [66]. This disease is characterized by the accumulation of surfactant which fills the terminal airways and alveoli, thereby impairing gas exchange and resulting in respiratory insufficiency. Based on the fact that this could not be visualized in other patients with non-PAP interstitial lung diseases, it was seen as an endomicroscopic characteristic strongly suggestive for the diagnosis of PAP. In theory, *in vivo* comparative studies on confocal alveolar imaging in sarcoidosis patients could identify new pulmonary phenotypes based on endomicroscopic characteristics.

The benefit of searching for causative agents and new clinical phenotypes in sarcoidosis was already demonstrated by Müller-Quernheim and colleques. In a group of 536 sarcoidosis patients from Germany and Israel 34 patients (6.3%) were diagnosed with chronic beryllium disease (CBD) instead of sarcoidosis after evaluation of beryllium exposure [67].

Finally, the results presented in chapter 5 deserve further attention. The suggested decrease in IL-23 production capacity in peripheral blood mononuclear cells of sarcoidosis patients is interesting based on its role in the development of Th-17cells.

It has already been demonstrated that a polymorphism in the receptor for IL-23 influences disease course in sarcoidosis [68]. Whether Th-17 cells are involved in sarcoidosis will certainly be determined in the near future.

The data presented in this thesis will hopefully stimulate others to further elucidate the role of innate immunity in sarcoidosis and search for new disease phenotypes in order to achieve a better understanding of this complex but intriguing disease.

Reference List

1. Takeda, K. & Akira, S. Toll-like receptors in innate immunity. *Int. Immunol.* **17**, 1-14 (2005).
2. Dubaniewicz, A., Kampfer, S. & Singh, M. Serum anti-mycobacterial heat shock proteins antibodies in sarcoidosis and tuberculosis. *Tuberculosis. (Edinb.)* **86**, 60-67 (2006).
3. Arbour, N. C. *et al.* TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. *Nat. Genet.* **25**, 187-191 (2000).
4. Bafica, A. *et al.* TLR9 regulates Th1 responses and cooperates with TLR2 in mediating optimal resistance to Mycobacterium tuberculosis. *J. Exp. Med* **202**, 1715-1724 (2005).
5. Romics, L., Jr. *et al.* Toll-like receptor 2 mediates inflammatory cytokine induction but not sensitization for liver injury by Propioni- bacterium acnes. *J. Leukoc. Biol.* **78**, 1255-1264 (2005).
6. Yim, J. J. *et al.* The association between microsatellite polymorphisms in intron II of the human Toll-like receptor 2 gene and tuberculosis among Koreans. *Genes Immun.* **7**, 150-155 (2006).
7. Pabst, S. *et al.* Toll-like receptor (TLR) 4 polymorphisms are associated with a chronic course of sarcoidosis. *Clin. Exp. Immunol.* **143**, 420-426 (2006).
8. Chang, Z. L. Important aspects of Toll-like receptors, ligands and their signaling pathways. *Inflamm. Res.* **59**, 791-808 (2010).
9. Baughman, R. P. & Iannuzzi, M. Tumour necrosis factor in sarcoidosis and its potential for targeted therapy. *BioDrugs. 2003* **17**, 425-431.
10. Takeuchi, O. *et al.* Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity.* **11**, 443-451 (1999).
11. Baldini, M. *et al.* A Polymorphism* in the 5' flanking region of the CD14 gene is associated with circulating soluble CD14 levels and with total serum immunoglobulin E. *Am. J. Respir. Cell Mol. Biol.* **20**, 976-983 (1999).
12. Inoue, Y. *et al.* CD14 -550 C/T, which is related to the serum level of soluble CD14, is associated with the development of respiratory syncytial virus bronchiolitis in the Japanese population. *J. Infect. Dis* **195**, 1618-1624 (2007).
13. Kalis, C. *et al.* Requirement for TLR9 in the immunomodulatory activity of Propionibacterium acnes. *J Immunol. 2005 Apr* **1;174**, 4295-4300.
14. Bafica, A. *et al.* TLR9 regulates Th1 responses and cooperates with TLR2 in mediating optimal resistance to Mycobacterium tuberculosis. *J Exp Med. 2005 Dec* **19;202**, 1715-1724.
15. Bertran, G., Arzt, E., Resnik, E., Mosca, C. & Nahmod, V. Inhibition of interferon gamma production by peripheral blood mononuclear leukocytes of patients with sarcoidosis. Pathogenic implications. *Chest* **101**, 996-999 (1992).
16. Daniele, R. P., Dauber, J. H. & Rossman, M. D. Immunologic abnormalities in sarcoidosis. *Ann. Intern. Med.* **92**, 406-416 (1980).

17. Goodwin, J. S., DeHoratius, R., Israel, H., Peake, G. T. & Messner, R. P. Suppressor cell function in sarcoidosis. *Ann. Intern. Med.* **90**, 169-173 (1979).

18. Rottoli, P., Muscettola, M., Grasso, G., Perari, M. G. & Vagliasindi, M. Imparied interferon-gamma production by peripheral blood mononuclear cells and effects of calcitriol in pulmonary sarcoidosis. *Sarcoidosis.* **10**, 108-114 (1993).

19. Miossec, P., Korn, T. & Kuchroo, V. K. Interleukin-17 and type 17 helper T cells. *N. Engl. J. Med.* **361**, 888-898 (2009).

20. Kirschning, C. J. & Schumann, R. R. TLR2: cellular sensor for microbial and endogenous molecular patterns. *Curr. Top. Microbiol. Immunol.* **270**, 121-144 (2002).

21. Guan, Y. *et al.* Human TLRs 10 and 1 share common mechanisms of innate immune sensing but not signaling. *J. Immunol.* **184**, 5094-5103 (2010).

22. Gupta, D., Agarwal, R., Aggarwal, A. N. & Jindal, S. K. Molecular evidence for the role of mycobacteria in sarcoidosis: a meta-analysis. *Eur. Respir. J.* **30**, 508-516 (2007).

23. Oswald-Richter, K. *et al.* Mycobacterial ESAT-6 and katG are recognized by sarcoidosis CD4+ T cells when presented by the American sarcoidosis susceptibility allele, DRB1*1101. *J. Clin. Immunol.* **30**, 157-166 (2010).

24. Oswald-Richter, K. A. *et al.* Cellular responses to mycobacterial antigens are present in bronchoalveolar lavage fluid used in the diagnosis of sarcoidosis. *Infect. Immun.* **77**, 3740-3748 (2009).

25. Song, Z. *et al.* Mycobacterial catalase-peroxidase is a tissue antigen and target of the adaptive immune response in systemic sarcoidosis. *J. Exp. Med.* **201**, 755-767 (2005).

26. Abe, C., Iwai, K., Mikami, R. & Hosoda, Y. Frequent isolation of Propionibacterium acnes from sarcoidosis lymph nodes. *Zentralbl. Bakteriol. Mikrobiol. Hyg. A* **256**, 541-547 (1984).

27. Eishi, Y. *et al.* Quantitative analysis of mycobacterial and propionibacterial DNA in lymph nodes of Japanese and European patients with sarcoidosis. *J. Clin. Microbiol.* **40**, 198-204 (2002).

28. McCaskill, J. G. *et al.* Pulmonary immune responses to Propionibacterium acnes in C57BL/6 and BALB/c mice. *Am. J. Respir. Cell Mol. Biol.* **35**, 347-356 (2006).

29. Chen, E. S. *et al.* T cell responses to mycobacterial catalase-peroxidase profile a pathogenic antigen in systemic sarcoidosis. *J. Immunol.* **181**, 8784-8796 (2008).

30. Ebe, Y. *et al.* Proliferative response of peripheral blood mononuclear cells and levels of antibody to recombinant protein from Propionibacterium acnes DNA expression library in Japanese patients with sarcoidosis. *Sarcoidosis. Vasc. Diffuse. Lung Dis.* **17**, 256-265 (2000).

31. Tella, J. L., Lemus, J. A., Carrete, M. & Blanco, G. The PHA test reflects acquired T-cell mediated immunocompetence in birds. *PLoS. One.* **3**, e3295 (2008).

32. Grunewald, J. Review: role of genetics in susceptibility and outcome of sarcoidosis. *Semin. Respir. Crit Care Med* **31**, 380-389 (2010).

33. Baehner, R. L. & Nathan, D. G. Leukocyte oxidase: defective activity in chronic granulomatous disease. *Science* **155**, 835-836 (1967).

34. Kanazawa, N. *et al.* Early-onset sarcoidosis and CARD15 mutations with constitutive nuclear factor-kappaB activation: common genetic etiology with Blau syndrome. *Blood* **105**, 1195-1197 (2005).

35. Marks, D. J. *et al.* Defective acute inflammation in Crohn's disease: a clinical investigation. *Lancet. 2006 Feb* **25;367**, 668-678.

36. Bafica, A. *et al.* TLR9 regulates Th1 responses and cooperates with TLR2 in mediating optimal resistance to Mycobacterium tuberculosis. *J Exp Med. 2005 Dec* **19;202**, 1715-1724.

37. Ogus, A. C. *et al.* The Arg753GLn polymorphism of the human toll-like receptor 2 gene in tuberculosis disease. *Eur Respir J. 2004 Feb* **23**, 219-223.

38. Yim, J. J. *et al.* The association between microsatellite polymorphisms in intron II of the human Toll-like receptor 2 gene and tuberculosis among Koreans. *Genes Immun. 2006 Mar* **7**, 150-155.

39. Barreiro, L. B. *et al.* Promoter variation in the DC-SIGN-encoding gene CD209 is associated with tuberculosis. *PLoS. Med* **3**, e20 (2006).

40. Sapkota, B. R. *et al.* Association of TNF, MBL, and VDR polymorphisms with leprosy phenotypes. *Hum. Immunol.* **71**, 992-998 (2010).

41. Berrington, W. R. *et al.* Common polymorphisms in the NOD2 gene region are associated with leprosy and its reactive states. *J. Infect. Dis* **201**, 1422-1435 (2010).

42. Wiken, M., Grunewald, J., Eklund, A. & Wahlstrom, J. Higher monocyte expression of TLR2 and TLR4, and enhanced pro-inflammatory synergy of TLR2 with NOD2 stimulation in sarcoidosis. *J. Clin. Immunol.* **29**, 78-89 (2009).

43. Oswald-Richter, K. A. *et al.* Cellular responses to mycobacterial antigens are present in bronchoalveolar lavage fluid used in the diagnosis of sarcoidosis. *Infect. Immun.* **77**, 3740-3748 (2009).

44. Chen, E. S. *et al.* Serum amyloid A regulates granulomatous inflammation in sarcoidosis through Toll-like receptor-2. *Am. J. Respir. Crit Care Med.* **181**, 360-373 (2010).

45. Ehrt, S. *et al.* Reprogramming of the macrophage transcriptome in response to interferon-gamma and Mycobacterium tuberculosis: signaling roles of nitric oxide synthase-2 and phagocyte oxidase. *J. Exp. Med* **194**, 1123-1140 (2001).

46. de Beer, F. C., Nel, A. E., Gie, R. P., Donald, P. R. & Strachan, A. F. Serum amyloid A protein and C-reactive protein levels in pulmonary tuberculosis: relationship to amyloidosis. *Thorax* **39**, 196-200 (1984).

47. Drake, W. P. *et al.* Cellular recognition of Mycobacterium tuberculosis ESAT-6 and KatG peptides in systemic sarcoidosis. *Infect. Immun.* **75**, 527-530 (2007).

48. van Ingen, J., de Zwaan, R., Dekhuijzen, R., Boeree, M. Region of difference 1 in nontuberculous Mycobacterium species adds a phylogenetic and taxonomical character. *J. Bacteriol.* **191**, 5865-5867 (2009).

49. van Ingen. J. *et al.* [Nontuberculous mycobacteria: clinically relevant]. *Ned. Tijdschr. Geneeskd.* **154**, A1178 (2010).

50. Khoor, A., Leslie, K. O., Tazelaar, H. D., Helmers, R. A. & Colby, T. V. Diffuse pulmonary disease caused by nontuberculous mycobacteria in immunocompetent people (hot tub lung). *Am. J. Clin. Pathol.* **115**, 755-762 (2001).

51. Veltkamp, M. *et al.* Toll-like receptor (TLR)-9 genetics and function in sarcoidosis. *Clin. Exp. Immunol.* (2010).

52. Miyara, M. *et al.* The immune paradox of sarcoidosis and regulatory T cells. *J. Exp. Med* **203**, 359-370 (2006).

53. Mroz, R. M., Korniluk, M., Stasiak-Barmuta, A., Ossolinska, M. & Chyczewska, E. Increased levels of Treg cells in bronchoalveolar lavage fluid and induced sputum of patients with active pulmonary sarcoidosis. *Eur. J. Med Res.* **14 Suppl 4**, 165-169 (2009).

54. Minami, J. *et al.* Pulmonary granulomas caused experimentally in mice by a recombinant trigger-factor protein of Propionibacterium acnes. *J. Med Dent. Sci.* **50**, 265-274 (2003).

55. Swanborg, R. H. Experimental autoimmune encephalomyelitis in the rat: lessons in T-cell immunology and autoreactivity. *Immunol. Rev.* **184**, 129-135 (2001).

56. Goulvestre, C., Batteux, F. & Charreire, J. Chemokines modulate experimental autoimmune thyroiditis through attraction of autoreactive or regulatory T cells. *Eur. J. Immunol.* **32**, 3435-3442 (2002).

57. Teuscher, C., Hickey, W. F., Grafer, C. M. & Tung, K. S. A common immunoregulatory locus controls susceptibility to actively induced experimental allergic encephalomyelitis and experimental allergic orchitis in BALB/c mice. *J. Immunol.* **160**, 2751-2756 (1998).

58. Hiramatsu, J. *et al.* Propionibacterium acnes DNA detected in bronchoalveolar lavage cells from patients with sarcoidosis. *Sarcoidosis. Vasc. Diffuse. Lung Dis* **20**, 197-203 (2003).

59. Jung, Y. S. *et al.* Propionibacterium acnes acts as an adjuvant in in vitro immunization of human peripheral blood mononuclear cells. *Biosci. Biotechnol. Biochem.* **71**, 1963-1969 (2007).

60. Hirsch, J. G. *et al.* The Kveim test. *N. Engl. J. Med* **284**, 1326-1328 (1971).

61. Richter, E. *et al.* Analysis of the Kveim-Siltzbach test reagent for bacterial DNA. *Am. J. Respir. Crit Care Med* **159**, 1981-1984 (1999).

62. Sabounchi-Schutt, F., Astrom, J., Hellman, U., Eklund, A. & Grunewald, J. Changes in bronchoalveolar lavage fluid proteins in sarcoidosis: a proteomics approach. *Eur. Respir. J.* **21**, 414-420 (2003).

63. Zissel, G., Prasse, A. & Muller-Quernheim, J. Immunologic response of sarcoidosis. *Semin. Respir. Crit Care Med* **31**, 390-403 (2010).

64. Statement on sarcoidosis. Joint Statement of the American Thoracic Society (ATS), the European Respiratory Society (ERS) and the World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG) adopted by the ATS Board of Directors and by the ERS Executive Committee, February 1999. *Am. J. Respir. Crit Care Med.* **160**, 736-755 (1999).

65. Drent, M. *et al.* Sarcoidosis: assessment of disease severity using HRCT. *Eur. Radiol.* **13**, 2462-2471 (2003).

66. Salaun, M., Roussel, F., Hauss, P. A., Lachkar, S. & Thiberville, L. In vivo imaging of pulmonary alveolar proteinosis using confocal endomicroscopy. *Eur. Respir. J.* **36**, 451-453 (2010).

67. Muller-Quernheim, J., Gaede, K. I., Fireman, E. & Zissel, G. Diagnoses of chronic beryllium disease within cohorts of sarcoidosis patients. *Eur. Respir. J.* **27**, 1190-1195 (2006).

68. Fischer, A. *et al.* Association of IBD Risk Loci with Sarcoidosis and its Acute and Chronic Subphenotypes. *Eur. Respir. J.* (2010).

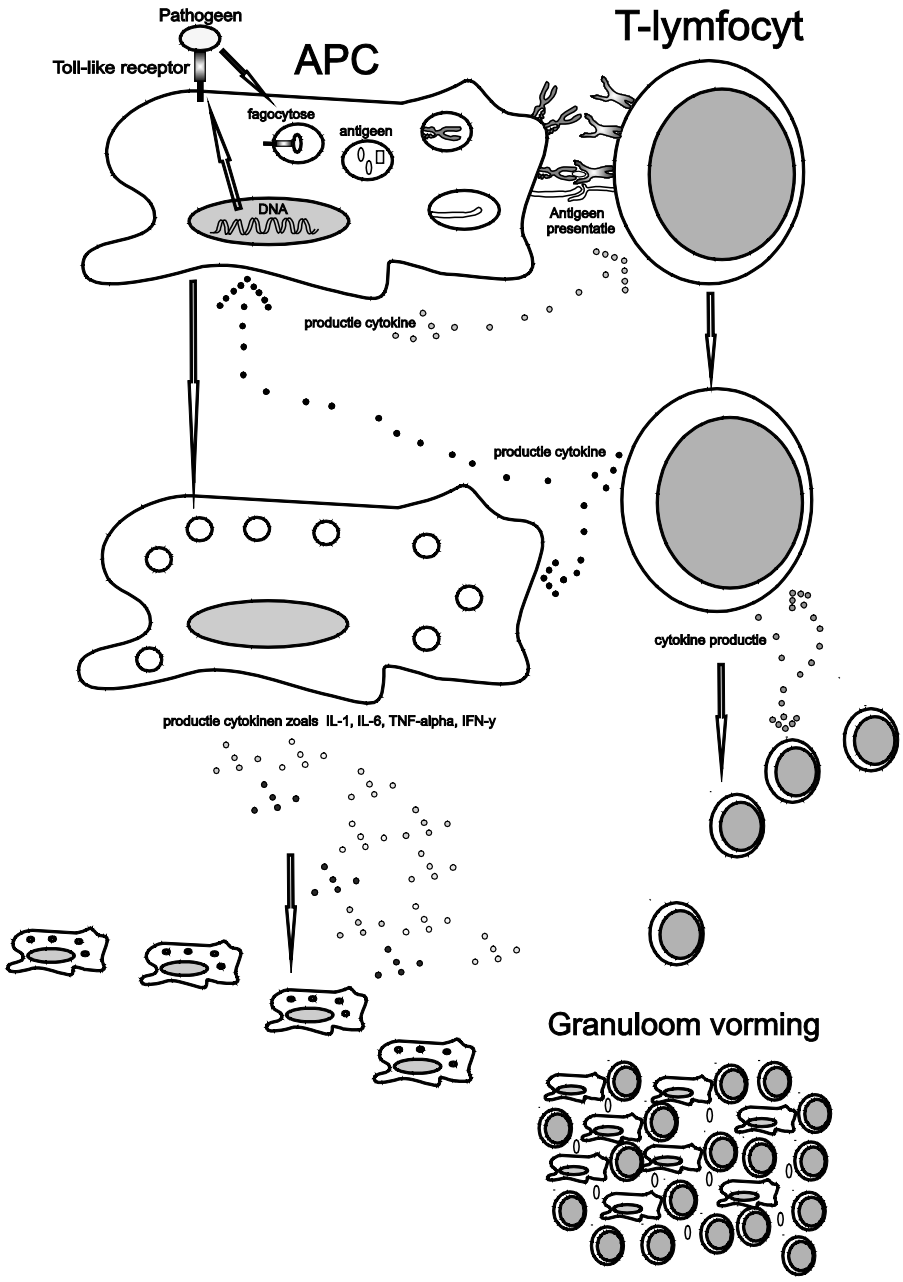
9

Dutch summary/
Nederlandse samenvatting

Inleiding

Sarcoïdose, ook wel de ziekte van Besnier-Boeck genoemd, is een ontstekingsziekte die zich kenmerkt door opeenhoping van groepen ontstekingscellen tot zogenaamde granulomen. De ziekte kan zich op alle leeftijden openbaren, echter op kinderleeftijd is het zeer zeldzaam. Het merendeel van de patiënten is tussen de 20 en 40 jaar oud. Over het algemeen kent de ziekte een gunstig beloop waarbij ongeveer 70% van de patiënten binnen 2 tot 3 jaar ziektevrij is. Bij de resterende groep is er sprake van chronische ziekte met in ongeveer 5% een fatale afloop, meestal door problemen met de ademhaling of hartritmestoornissen. Voor de individuele patiënt is het moeilijk om ten tijde van diagnose al een voorspelling te doen over het beloop van de ziekte, wat een grote onzekerheid met zich meebrengt.

Het belangrijkste kenmerk van de ziekte is, zoals al eerder genoemd, het optreden van de zogenaamde granulomen. Deze kunnen zich overal in het lichaam vormen, echter meestal zijn ze gelokaliseerd in de longen, lymfklieren of de huid. Indien in grote getalen aanwezig, kunnen deze granulomen weefselschade of het dysfunctioneren van organen veroorzaken. Van oudsher is de functie van een granuloom om een bepaalde indringer (pathogeen), zoals een bacterie of parasiet, te isoleren en op te ruimen. Blijkt dit niet mogelijk, dan zorgt het granuloom ervoor dat het pathogeen wordt afgesloten van de omgeving en geen schade meer kan berokkenen. Voordat er zich in het lichaam granulomen vormen is er al een hele cascade van afweerreacties, of immuunrespons, in gang gezet. Belangrijke cellen in deze immuunrespons zijn Antigeen Presenterende Cellen (APCs) en T-lymfocyten (figuur 1). De APCs zijn de cellen die als eerste bepaalde pathogenen als lichaamsvreemd herkennen en de immuunrespons in gang zetten. Het pathogeen wordt naar de binnenkant van de APC gebracht (fagocytose) en aldaar in stukjes (antigenen) geknipt. Deze antigen worden vervolgens weer op het celmembraan gebracht en gepresenteerd aan T-lymfocyten. Indien er T-lymfocyten zijn die de gepresenteerde antigenen ook als lichaamsvreemd herkennen wordt de immuunrespons voortgezet. Er zijn 2 belangrijke immuunresponsen: de cellulaire imuunrespons en de humorale immuunrespons. De cellulaire immuunrespons zorgt voor de afweer tegen intracellulaire bacteriën of virussen, de humorale response zorgt voor extracellulaire pathogenen zoals bacteriën en sommige parasieten.



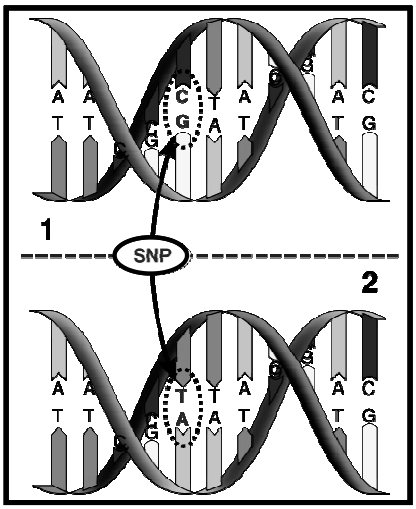
Figuur 1. Schematisch overzicht van antigeenherkenning, antigeenpresentatie, activatie van T-lymphocyten en granuloomvorming.

In het DNA van de Antigeen Presenterende Cel (APC) ligt onder andere de genetische informatie voor de vorming van Toll-like receptoren (TLR). Wanneer een TLR op het celoppervlak een pathogeen herkent wordt deze naar de binnenkant van de APC gebracht (= fagocytose). Vervolgens wordt dit pathogeen in stukjes geknipt tot antigenen. Deze antigenen worden mbv andere eiwitten op het celmembraan van de APC gebracht en gepresenteerd aan T-lymfocyten (=antigeenpresentatie). Bij herkenning van het antigeen door een T lymfocyt wordt deze geactiveerd en zal de APC op haar beurt ook weer geactiveerd worden. De immuunrespons is hiermee in gang gezet. Er worden allerlei cytokinen geproduceerd waaronder interferon-gamma (IFN-γ) en Tumor Necrosis Factor-alpha (TNF-α). Wanneer de APC en T-lymfocyt niet in staat blijken om het pathogeen op te ruimen worden de zogenaamde granulomen gevormd. Deze bestaan onder andere uit de APCs, T-lymphocyten en het desbetreffende antigeen.

De laatste jaren is er veel onderzoek gedaan naar de eerste herkenning van pathogenen door Antigeen Presenterende Cellen. Het blijkt zo te zijn dat deze eerste interactie al invloed heeft op de mate en het type imuunrespons dat gestart gaat worden. Belangrijke eiwitten op het celmembraan (receptoren) van APCs die pathogenen kunnen herkennen zijn de zogenaamde Toll-like receptoren (TLR). Deze receptorfamilie bestaat uit 11 verschillende receptoren die elk bepaalde structuren herkennen die heel specifiek zijn voor pathogenen. Het zijn als het ware "grijparmpjes" op het celoppervlak van immuuncellen (figuur 1).
Een eventueel pathogeen voor sarcoïdose is tot op heden nog niet gevonden. Een gedachte is dat sarcoïdose niet 1 ziekte is, maar een groep van verschillende granulomateuze ziekten, ieder met hun eigen pathogeen. Dit is echter nog niet bewezen. Als mogelijke pathogenen voor sarcoïdose zijn *Mycobacteriën* en *Propionibacteriën* het meest waarschijnlijk op basis van bacteriële eigenschappen, klinisch beeld bij infecties en het aantonen van DNA of antigeenspecifieke T lymfocyten in patiënten. De meest bekende mycobacterie is *Mycobacterium tuberculosis* die de ziekte tuberculose kan veroorzaken. Echter, slechts 10% van alle mensen die besmet worden met deze bacterie ontwikkelt daadwerkelijk tuberculose. De meest bekende propionibacterie is *Propionibacterium acnes* die de ziekte acne kan veroorzaken. Vanaf de puberteit zit deze bacterie bij iedereen op de huid, echter kan hij ook gevonden worden in het bovenste gedeelte van de luchtwegen en darmen.

Doel van het proefschrift

In dit proefschrift is onderzocht of variaties in de genen coderend voor Toll-like receptoren van invloed zijn op het krijgen van sarcoïdose of het verloop van de ziekte na het stellen van de diagnose. De variaties die onderzocht zijn worden gekenmerkt door het verschil van 1 nucleotide (nucleotiden zijn de bouwstenen van het menselijk DNA) op een bepaalde plek in een gen. Een gen is een gedeelte van het DNA dat de genetische informatie bevat om 1 bepaald eiwit te produceren. Elk eiwit heeft een bepaalde functie in het lichaam zoals bijvoorbeeld insuline (opname van glucose) en hemoglobine (binden van zuurstof). Geschat wordt dat het totale menselijke DNA ongeveer 25.000 verschillende genen bevat. De kleine 1-punts variaties die onderzocht zijn in dit proefschrift worden single nucleotide polymorphisms (SNPs) genoemd. In figuur 2 wordt dit schematisch weergegeven. De manier waarop de nucleotiden gerangschikt zijn naast elkaar geven informatie over hoe het eiwit, waar het gen voor codeert, er uit zal zien. SNPs kunnen leiden tot structurele veranderingen van het coderende eiwit waardoor de functie van het eiwit gestoord raakt of de expressie verhoogd of verlaagd is. Er is gekeken naar de genen van specifieke Toll-like receptoren betrokken bij de herkenning van bacteriën gezien het feit dat deze groep van pathogenen sarcoïdose zou kunnen veroorzaken. Verder is onderzocht of er aanwijzingen zijn dat mycobacteriën of propionibacteriën ook een rol kunnen spelen bij het veroorzaken van sarcoïdose in Nederland.



Figuur 2. Voorbeeld van een single nucleotide polymorphism (SNP). Persoon 1 heeft op een bepaalde locatie in het DNA een bouwsteen van het type C op de ene streng en type G op de andere streng (omcirkeld met stippellijn). Persoon 2 heeft op die exacte locatie in het DNA een bouwsteen type T ipv C op de ene streng en type A ipv G op de andere streng. Door het verschil van bouwstenen op die ene plek verandert de genetische informatie die zorgt voor het bouwen van een bepaald eiwit waar dit gen voor codeert. De opbouw van dit eiwit kan hierdoor veranderen evenals de functie van dit eiwit.

Hoofdstuk 2: Het belangrijkste functionele polylmorfisme in het gen coderend voor Toll-like receptor 4 is niet geassocieerd met de vatbaarheid voor sarcoïdose of het beloop van de ziekte.

In dit hoofstuk wordt gekeken naar het belangrijkste polymorfisme in het gen coderend voor Toll-like receptor 4 (TLR4), Asp299Gly. TLR4 herkent onder andere het eiwit LPS (LipoPolySaccharide), onderdeel van de celwand van een grote groep bacteriën (Gram-negatieve bacteriën genoemd). Bij verandering van nucleotide A naar nucleotide G verandert de structuur van TLR-4 met een verminderd functioneren tot gevolg. Het polymorfisme wordt onderzocht in 156 patiënten met sarcoïdose en 200 gezonde controles. De genetische variaties van dit polymorfisme verschillen niet tussen de beide groepen. Ook wanneer gekeken wordt naar patiënten met een gunstig beloop in vergelijking met patiënten met een chronische vorm van sarcoïdose worden geen verschillen waargenomen. Het feit dat dit polymorfisme geen invloed heeft op de ziekte zou verklaard kunnen worden door het feit dat

de eerder genoemde mogelijke bacteriele verwekkers van sarcoïdose geen deel uitmaken van de groep Gram-negatieve bacteriën. Tevens laten de resultaten zien dat de associatie tussen Asp299Gly en sarcoïdose gevonden door een Duitse onderzoeksgroep gebaseerd is op afwijkende genetische variaties in de controlegroep in plaats van de patiëntengroep.

Hoofdstuk 3: De relatie tussen een promoterpolymorfisme en repeatpolymorfismen in het gen coderend voor Toll-like receptor (TLR) 2; functionele effecten en klinische relevantie voor sarcoïdose.

In dit hoofstuk worden 3 SNPs en 1 repeatpolymorfisme onderzocht in het gen coderend voor TLR-2. Deze receptor is betrokken bij de immuunrespons tegen zowel *Mycobacterium tuberculosis* als *Propionibacterium acnes*. Het repeatpolymorfisme is een gedeelte van het TLR2 gen dat bestaat uit een herhaling van de nucleotidecombinatie GT (GTGTGTGT etc). Het aantal keren dat deze combinatie herhaald wordt, het aantal GT-repeats, verschilt tussen individuen. De lengte van deze sequentie heeft mogelijk invloed op de functie van TLR-2. De 3 SNPs hebben elk ook een effect op de expresssie en/of functie van TLR-2. In totaal worden er 419 patiënten en 196 gezonde controles onderzocht. De 419 patiënten zijn verdeeld in een studiecohort (n=165) en een bevestigingscohort (n=254). Er worden geen verschillen in genetische variaties gevonden tussen de patiënten en de gezonde controles. Echter, wanneer in het studiecohort gekeken wordt naar de patiënten met een gunstig beloop van de ziekte in vergelijking met chronische sarcoïdosepatiënten worden er wel verschillen gevonden. De genetische variatie AA van het promoterpolymorfisme (AA-genotype)wordt significant vaker gezien bij patiënten met een chronisch verloop. Verder bleek dat het aantal GT-repeats bij deze groep ook lager was. Opvallend is dat er een correlatie gevonden is tussen het genotype van het promoterpolymorfisme en het aantal GT-repeats: het aantal GT-repeats in mensen met het AA-genotype is significant lager in vergelijking met mensen die het GG-genotype hebben. Om een eventueel functioneel effect van deze genetische variatie aan te tonen werd bloed van 15 gezonde donoren gestimuleerd met specifieke TLR-2 antigenen om de receptor te activeren. Van te voren werd per

donor het aantal GT-repeats bepaald. De resultaten van dit experiment laten zien dat donoren met weinig GT-repeats een hogere productie hebben van het cytokine TNF-alpha in vergelijking met donoren met een hoog aantal GT-repeats, op een gelijke stimulus van TLR-2. Het cytokine TNF-alpha is belangrijk bij de vorming van granulomen en is vaak verhoogd bij patiënten met chronische sarcoïdose. Het feit dat juist in deze groep genotype AA van het promoterpolymorfisme vaker voorkomt zou een gedeeltelijke verklaring kunnen zijn voor het feit dat er bij chronische patiënten meer TNF-alpha aanwezig is. Anders gezegd, bij sarcoïdosepatiënten zou de aanwezigheid van het AA genotype in de promoter van TLR2 de kans groter kunnen maken op het ontwikkelen van chronische ziekte omdat er relatief veel TNF-alpha geproduceerd wordt bij stimulatie van TLR-2. Om de gevonden genetische associatie te bevestigen werd een andere en onafhankelijke groep van sarcoïdosepatiënten bekeken. In deze groep werden geen verschillen gevonden tussen patiënten met een mild beloop en patiënten met een chronisch beloop van de ziekte. Dit suggereert dat de associatie in het studiecohort gebaseerd is op louter toeval. Het blijft echter moeilijk om verschillende groepen met elkaar te vergelijken omdat we de oorzaak van de ziekte niet kennen. Misschien zijn er in het studiecohort wel relatief meer patiënten die een bacteriële verwekker hebben dan in het bevestigingscohort. Verder onderzoek met meer patiënten moet duidelijk maken of de gevonden associatie enige betekenis heeft of inderdaad berust op toeval.

Hoofdstuk 4. Genetica van CD14 in sarcoïdose.

In dit hoofdstuk worden 4 SNPs in het gen coderend voor CD14 onderzocht. CD14 is van oudsher de klassieke receptor voor het herkennen van LPS (lipopolysaccharide). De reden om dit gen te onderzoeken is het feit dat CD14 een receptorcomplex kan vormen met TLR-2 om zo bij te dragen aan het herkennen van antigenen door TLR-2. Twee van de onderzochte SNPs hebben mogelijk invloed op de mate van expressie van CD14. In totaal zijn 533 patiënten met sarcoïdose en 447 gezonde personen onderzocht. De resultaten laten zien dat een bepaald haplotype (een haplotype is een vaste combinatie van een aantal SNPs verspreid over een stuk DNA) vaker

voorkomt bij patiënten met sarcoïdose. Interessant is het feit dat dit haplotype met name vaker voorkomt bij patiënten met een chronisch verloop van sarcoïdose, zoals ook al gezien werd voor een bepaald genotype in TLR-2. De gedachte is dan ook dat niet alleen genetische variatie in TLR-2, maar ook in co-receptoren van TLR-2 de kans op chronische sarcoïdose vergroten.

Hoofdstuk 5: Genetica en functie van Toll-like receptor (TLR) 9 in sarcoïdose.

Dit hoofstuk beschrijft het onderzoek naar 4 SNPs in het gen coderend voor TLR9. TLR9 herkent bepaalde DNA structuren die karakteristiek zijn voor bacteriën. Deze receptor is betrokken bij de immuunrespons tegen zowel *Mycobacterium tuberculosis* als *Propionibacterium acnes*. Er werden 185 gezonde controles en 533 patiënten onderzocht in deze studie, waarbij de patiënten verdeeld werden in een studiecohort (n=150) en een bevestigingscohort (n=383). Er werden geen verschillen waargenomen in de 4 SNPs tussen patiënten en gezonde controles. Ook bij het vergelijken van verschillende groepen sarcoïdose patiënten onderling werden geen verschillen gezien. Om uit te sluiten dat er geen sarcoïdose-specifieke mutaties bestaan in het gen coderend voor TLR-9 zijn de belangrijkste gedeelten van dit gen nucleotide voor nucleotide bekeken (sequencing). Dit is gedaan in 13 sarcoïdose patiënten en 7 patiënten met het syndroom van Löfgren. Er zijn geen sarcoïdose-specifieke mutaties gevonden. Het feit dat er geen genetische verschillen gevonden zijn in TLR9 tussen patiënten en controles wil niet direct zeggen dat de receptor in beide groepen evengoed werkt. Zodoende is besloten om de de receptorfunctie van TLR-9 te testen door bloed van zowel patiënten als gezonde controles te stimuleren met TLR-9 agonisten (stoffen die de receptor activeren). Sarcoïdose patiënten hebben een duidelijk verminderde productie van interferon-gamma (IFN-γ) op verschillende stimuli. IFN-γ is een belangrijk cytokine voor het adequaat bestrijden van intracellulaire pathogenen. De verminderde IFN-γ productiecapaciteit van T-cellen uit het bloed van patiënten lijkt echter niet verklaard te worden door een afwijkende functie van TLR-9. Er werd tevens een vermindere productiecapaciteit van IL-23 gevonden bij de patiënten. IL-23 is ook een belangrijk cytokine in de bestrijding van intracellulaire pathogenen. Het verschil in IL-23

productie lijkt wel voor een deel te wijten aan een vermindere functie van TLR-9. Deze resultaten zouden kunnen suggereren dat sarcoïdosepatiënten een verminderde functie hebben van TLR-9 hetgeen zorgt voor een minder adequate immuunrespons. Gezien de kleine aantallen patiënten en controles blijft dit speculeren en is het niet mogelijk om harde conclusies te trekken. Vervolgonderzoek met meer patiënten en het gebruik van T-cellen afkomstig uit de longen zal duidelijkheid moeten geven of er daadwerkelijk een verminderde functie is van TLR-9 in sarcoïdosepatiënten.

Hoofdstuk 6: Genetische variatie in genen-cluster TLR10-TLR1-TLR6 is geassocieerd met een chronisch verloop van de ziekte sarcoïdose.

In dit hoofdstuk worden 8 SNPs bekeken in 3 verschillende genen, elk coderend voor een TLR. Deze 3 genen liggen naast elkaar op chromosoom 4 (zijn "geclusterd"). Deze 3 genen zijn interessant omdat er aanwijzingen zijn dat zowel TLR-10, TLR-1 als TLR-6 samenwerken met TLR-2 bij het herkennen van pathogenen (ze hebben de functie van "co-receptor"). Een afwijking in een van deze genen zou dus een veranderde functie van TLR-2 kunnen bewerkstelligen. Er zijn 533 patiënten en 447 gezonde controles onderzocht. Van de 8 SNPs zijn haplotypen gemaakt (een haplotype is een vaste combinatie van een aantal SNPs verspreid over een bepaald stuk DNA). Het haplotype dat bij gezonde mensen het meeste voorkomt (42% van alle mensen hebben dit haplotype), komt veel minder vaak voor bij patiënten met een chronische vorm van sarcoïdose (30%). Aanwezigheid van dit haplotype zou dus beschermend kunnen werken tegen het optreden van chronische ziekte bij patiënten die reeds sarcoïdose hebben. Opvallend is wederom dat bepaalde genetische variaties gevonden worden bij patiënten met een chronische vorm van de ziekte. Dit was ook al het geval voor TLR-2 en CD14. De volgende stap in het onderzoek zal moeten zijn of de gevonden genetische variaties in het gen-cluster TLR1-TLR1-TLR-6 ook daadwerkelijk invloed hebben op de werking van TLR-2 receptor.

Hoofstuk 7: T-cel responsen op propionibacteriën en mycobacteriën in Nederlandse sarcoïdosepatiënten.

In dit hoofstuk wordt allereerst gekeken naar de respons van T lymphocyten in aanwezigheid van mycobacteriële antigenen. Een snelle reactie van T cellen op deze antigenen geeft aan dat er zogenaamde geheugen T cellen (memory T cells) aanwezig zijn hetgeen duidt op een eerdere besmetting in het verleden. De vraag is of besmetting met mycobacteriën in het verleden bij Nederlandse sarcoïdose patiënten vaker heeft plaatsgevonden in vergelijking met gezonde controles. Dit zou een aanwijzing kunnen zijn dat deze bacteriën mogelijke verwekkers zijn van sarcoïdose. Er zijn 2 groepen patiënten bekeken waarbij het percentage met een eerdere besmetting respectievelijk 6.7 en 4.8% is, hetgeen iets hoger lijkt dan het percentage besmetting in gezonde controles. Echter, de resultaten laten zien dat besmetting met mycobacteriën geen grote rol speelt in het krijgen van sarcoïdose in Nederland. Deze resultaten passen bij de gedachte dat indien *Mycobacterium tuberculosis* sarcoïdose zou kunnen veroozaken, het te verwachten is dat in landen waar veel tuberculose voorkomt er ook relatief veel patiënten met sarcoïdose zullen zijn. Dit blijkt echter niet zo te zijn. Er zijn 3 andere landen in de wereld waar soortgelijk ondezoek is verricht. In Zweden en Amerika is het aantal patiënten met sarcoïdose dat in het verleden besmet is met *Mycobacterium tuberculosis* relatief hoog (geschat op 50%). Echter, in beiden landen komt tuberculose, net als in Nederland, relatief weinig voor. In Japan, waar tuberculose relatief vaak voorkomt, is het percentage sarcoïdosepatiënten dat ooit besmet is met *Mycobacterium tuberculosis* laag, namelijk 3%. Het is niet duidelijk waardoor deze verschillen worden veroorzaakt. Verder onderzoek in andere landen en meer patiënten zal duidelijkheid moeten geven over dit vraagstuk.

In het hoofdstuk is tevens gekeken naar de aanwezigheid van eventuele Memory T cellen reagerend op *P. acnes* of *P. granulosum*. In tegenstelling tot mycobacteriën maken deze bacteriën deel uit van de normale bacteriële kolonie die mensen bij zich dragen vanaf de puberteit. Uit de experimenten blijkt dat zowel alle gezonde controles als alle patiënten een T lymfocyten respons hebben op beide

bacteriën. Om er zeker van te zijn dat de resultaten daadwerkelijk berusten op memory T cellen zijn de experimenten ook uitgevoerd met T cellen afkomstig van navelstrengsbloed van pasgeboren babies. In dit navelstrengsbloed zitten namelijk geen memory T cellen omdat bij een normale zwangerschap het kind niet in aanraking komt met bacteriën. De resultaten laten echter zien dat ook de T lymphocyten uit navelstrengsbloed reageren op de bacteriën, hetgeen impliceert dat deze bacteriën in staat zijn om alle T-cellen te stimuleren. Gezien het feit dat zowel gezonde mensen als patiënten reageren, maakt het moeilijk om een verschil in respons aan te tonen. Dit experiment geeft ons dus ook geen antwoord op de vraag bij welk percentage patiënten propionibacteriën een verwekker zouden kunnen zijn. De aanwijzingen dat propionibacteriën sterke aspecifieke stimuli zijn voor T cellen is interessant. In theorie zou het zo kunnen zijn dat deze bacteriën niet de oorzaak zijn van sarcoïdose, maar de aanwezige immuunreactie tegen een ander antigen versterken door hun stimulerende effect op T lymphocyten.

Conclusie:

Het doel van dit proefschrift was om te kijken of genetische veranderingen in Toll-like receptoren een rol spelen in de vatbaarheid voor sarcoïdose of van invloed zijn op het verloop van de ziekte. Tevens is gekeken of er aanwijzingen zijn dat mycobacteriën en propionibacteriën een rol kunnen spelen bij het ontstaan van sarcoïdose in de Nederlandse populatie.

Een van moeilijkheden in genetisch onderzoek bij sarcoïdose is het feit dat de oorzaak niet bekend is. In theorie zou het een ziekte kunnen zijn met slechts één oorzaak of een groep van overeenkomstige ziekten met bijvoorbeeld 10 verschillende oorzaken. Hierdoor is het moeilijk om verschillende cohorten van patiënten goed te definiëren met het oog op onderlinge vergelijking, en het verifiëren van gevonden associaties. Ook zijn er nog maar weinig duidelijk verschillende klinische fenotypen bekend. Een fenotype is een bepaalde kenmerkende presentatie en/of beloop van de ziekte. Een goed gedefinieerd fenotype binnen

de sarcoïdosepopulatie is het syndroom van Löfgren, gekenmerkt door koorts, huidafwijkingen, gewrichtsontstekingen en vergroting van lymfklieren tussen beide longen (hilaire en mediastinale lymfklieren). Andere sarcoïdose patiënten worden in de literatuur vaak beschreven als "non-Löfgren" sarcoïdose. In deze laatste groep wordt in dit proefschrift een onderverdeling gemaakt tussen acute en vaak gunstig verlopende sarcoïdose en chronische sarcoïdose. Deze indeling is gebaseerd op de verdeling van afwijkingen op een longfoto (thoraxfoto) hetgeen vatbaar lijkt voor subjectiviteit. Er is nu veel onderzoek gaande om ziekteactiviteit beter in kaart te brengen waardoor in de toekomst het makkelijker zal worden om verschillende groepen beter te definiëren. Ook beeldvormende technieken zoals High Resolution Computer Tomography (HRCT) en Positive Emmission Tomography (PET) zullen hier ongetwijfeld aan bijdragen.

In dit proefschrift zijn 7 genen onderzocht bestaande uit 6 TLRs en CD14. Bij 4 van de 7 genen worden sterke en/of zwakke associaties gevonden met de vatbaarheid voor sarcoïdose of het verloop van de ziekte. Deze 4 genen hebben met elkaar gemeen dat ze een rol spelen bij de herkenning van pathogenen door TLR-2. Allereerst is er een genetische associatie gevonden tussen TLR-2 zelf en de chronische vorm van sarcoïdose. De data afkomstig uit de in-vitro experimenten beschreven in hoofstuk 3 geven een mogelijke verklaring voor het feit dat bij patiënten met chronische sarcoïdose er vaak een hoge concentratie van het cytokine TNF-alpha gevonden wordt. De gedachte is dat er bij sarcoïdose sprake is van persisterende antigenpresentatie vanuit de granulomen wat zorgt voor continue activiteit van het aangeboren immuunsysteem met bijbehorende cytokinenprofielen. Deze hypothese wordt ondersteund door studies waaruit blijkt dat de expressie van TLR-2 in sarcoïdose patiënten verhoogd is en het feit dat TLR-2 betrokken is bij de herkenning van zowel *Mycobacterium tuberculosis* als *Propionibacterium acnes*. Echter, de gevonden associatie kon niet worden bevestigd in een onafhankelijk cohort van patiënten, dus meer onderzoek in goed omschreven groepen patiënten is nodig om harde conclusies te kunnen trekken.De genetische associaties tussen TLR-10, TLR-1 en sarcoïdose beschreven in hoofdstuk 6 passen wel weer bij de eerder genoemde hypothese. De associatie gevonden tussen zowel TLR-10 als TLR-1 suggereert dat

beide genen invloed hebben op de vatbaarheid van sarccoidose. Echter, bij het vergelijken van acute met chronische patiënten lijkt met name het ontbreken van het meest voorkomende haplotype bij de chronische patiënten te zorgen voor de associatie. Ook deze resultaten suggereren dat een afwijkende functie van TLR-2 een verhoogde kans geeft op het ontwikkelen van chronische ziekte. De associatie met CD14, beschreven in hoofdstuk 4, past ook bij de bovenstaande hypothese.

Bij onderzoek van TLR-9, ook belangrijk voor de herkennning van *M. tuberculosis* en *P.acnes*, werden geen genetische associaties gevonden. Wel zijn er aanwijzingen dat de functie van deze receptor bij patiënten mogelijk anders is in vergelijking met gezonde controles, ondanks het feit dat er geen genetische verschillen zijn gevonden.

Al met al laten de beschreven resultaten zien dat genetische variaties en/of functionele verschillen in TLRs betrokken bij de herkenning van onder andere *M. tuberculosis* en *P.acnes* waarschijnlijk invloed hebben op het klinisch beloop van sarcoïdose. Kennis over mechanismen die ten grondslag liggen aan het ontstaan van een ziekte of het ziektebeloop is essentieel om eventuele nieuwe behandelingen te ontwikkelen. Indien de associatie tussen TLR-2 en chronische sarcoïdose inderdaad bestaat zou het eventueel remmen van TLR-2 in deze patiëntengroep gunstig kunnen zijn met het oog op een verminderde TNF-alpha productie. Het verminderen van de hoeveelheid of werking van TNF-alpha bij patiënten met een chronische en ernstige vorm van sarcoïdose is al effectief gebleken. Middelen zoals Remicade (Infliximab) die TNF-alpha wegvangen uit het bloed worden sinds enkele jaren met succes aan sommige patiënten met sarcoïdose gegeven. Een groot probleem zal zijn om de goede patiënten te selecteren die baat kunnen hebben bij het remmen van TLR-2. Nieuwe studies waarbij per patiënt gekeken gaat worden wat de verwekker is van sarcoïdose lijken nodig om deze vraag te kunnen beantwoorden. Hopelijk zetten de resultaten beschreven in dit proefschrift anderen aan tot verder onderzoek naar de rol van TLR-2 in sarcoïdose. Bij andere aandoeningen zoals kanker zijn de laatste jaren al veelbelovende medicijnen voortgekomen uit immunologisch onderzoek. Laten we hopen dat dit bij sarcoïdose ook mogelijk is.

Publications

M.Veltkamp, G.T. Rijkers, D. Daniels-Hijdra, J.M.M. Bouwman, J.M.M. van den Bosch and J.C. Grutters. In vitro γ-interferon responses to *Mycobacterium tuberculosis*, *Propionibacterium acnes* and *P. granulosum* in Dutch sarcoidosis patients. Manuscript in preparation.

M.Veltkamp, C.H.M. van Moorsel, G.T. Rijkers, H.J.T. Ruven, J.C. Grutters and J.M.M. van den Bosch. Genetic variation in the Toll-like Receptor gene cluster (TLR10-TLR1-TLR6) influences disease course in sarcoidosis. Submitted.

M.Veltkamp, J.C. Grutters. *Hoofstuk 24: Een diffuus longbeeld*. Probleemgeoriënteerd denken in de longgeneeskunde. Een praktijkboek voor de opleiding en kliniek, blz 249-260.
de Tijdstroom, Utrecht, 2010. ISBN 9789058981769

M.Veltkamp, C.H.M. van Moorsel, G.T. Rijkers, H.J.T. Ruven, J.M.M. van den Bosch and J.C. Grutters. *Toll-like receptor (TLR)-9 genetics and function in sarcoidosis*. Clinical and Experimental Immunology, 2010; 162 (1) 68-74.

M. Heron, J.C. Grutters, H. van Velzen-Blad, M. Veltkamp, A.M.E. Claessen and J.M.M. van den Bosch. *Increased expression of CD16, CD69 and VLA-1 on blood monocytes in active sarcoidosis*. Chest 2008; 134(5): 1001-1008.

M.Veltkamp, J.C. Grutters, C.H.M. van Moorsel, G.T. Rijkers, H.J.T. Ruven, M. Drent and J.M.M. van den Bosch. *CD14 genetics in sarcoidosis; who's in control*. Sarcoidosis, Vasculitis and Diffuse Lung Diseases, 2007 Sep;24(2): 154-5.

M.Veltkamp, P.A.H.M. Wijnen, C.H.M. van Moorsel, G.T. Rijkers, H.J.T. Ruven, M. Heron, O. Bekers, A.M.E. Claessen, M. Drent, J.M.M. van den Bosch and J.C. Grutters. *Linkage between Toll-like receptor (TLR) 2 promoter and intron polymorphisms; functional effects and relevance to sarcoidosis*. Clinical and Experimental Immunology, 2007; 149 (3) 453-462.

M.Veltkamp, C.H.M. van Moorsel, G.T. Rijkers, H.J.T. Ruven, J.M.M. van den Bosch and J.C. Grutters. *Toll-like receptor (TLR) 4 polymorphism Asp299Gly is not associated with disease course in Dutch sarcoidosis patients.* Clinical and Experimental Immunology, 2006; 145 (2) 215-218.

M.Feenstra, M.Veltkamp, J. van Kuik, S. Wiertsema, P. Slootweg, J. van den Tweel, R. de Weger and M. Tilanus. *HLA class I expression and chromosomal deletions at 6p and 15q in head and neck squamous cell carcinomas.* Tissue Antigens. 1999 Sep;54(3): 235-245.

Dankwoord

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Curriculum Vitae

Marcel Veltkamp was born on July 24th 1976 in Holten, The Netherlands. From 1988 he received secondary education at Scholengemeenschap Holten, where he graduated (VWO) in 1994. In this year he started his study medical biology at the University of Utrecht and in 1997 he commenced his medical training at the same university. During his studies he performed research at the department of pathology (head: prof. dr. J. van den Tweel) and the department of immunology (head: prof. dr. J.C. Clevers). In 1999 he obtained his master's degree (MSc) in medical biology. In 2003, after an internship internal medicine at the University Teaching Hospital in Lusaka, Zambia (head: dr. P. Mwaba), he obtained his medical degree.

He worked as a resident at the department of internal medicine, Diakonessenhuis, Utrecht (head: dr. J.W.M. Hustinx) from October 2003 through December 2004. From January 2005 until March 2007 he worked as a research fellow at the department of pulmonology, St. Antonius Hospital, Nieuwegein (head: prof. dr. J.M.M. van den Bosch). The research performed in this period was the foundation of this thesis. In March 2007 he started his specialist training in respiratory medicine at the St. Antonius Hospital, Nieuwegein (head: prof. dr. J.M.M. van den Bosch, succeeded by prof. dr. J.C. Grutters). As a part of this training he worked as a specialist registrar at the department of internal medicine of the Meander Medisch Centrum, Amersfoort (March 2007-August 2008; head: dr. C.A.J.M. Gaillard).

Marcel is married to Annet Hose (2008) and they have 2 children.