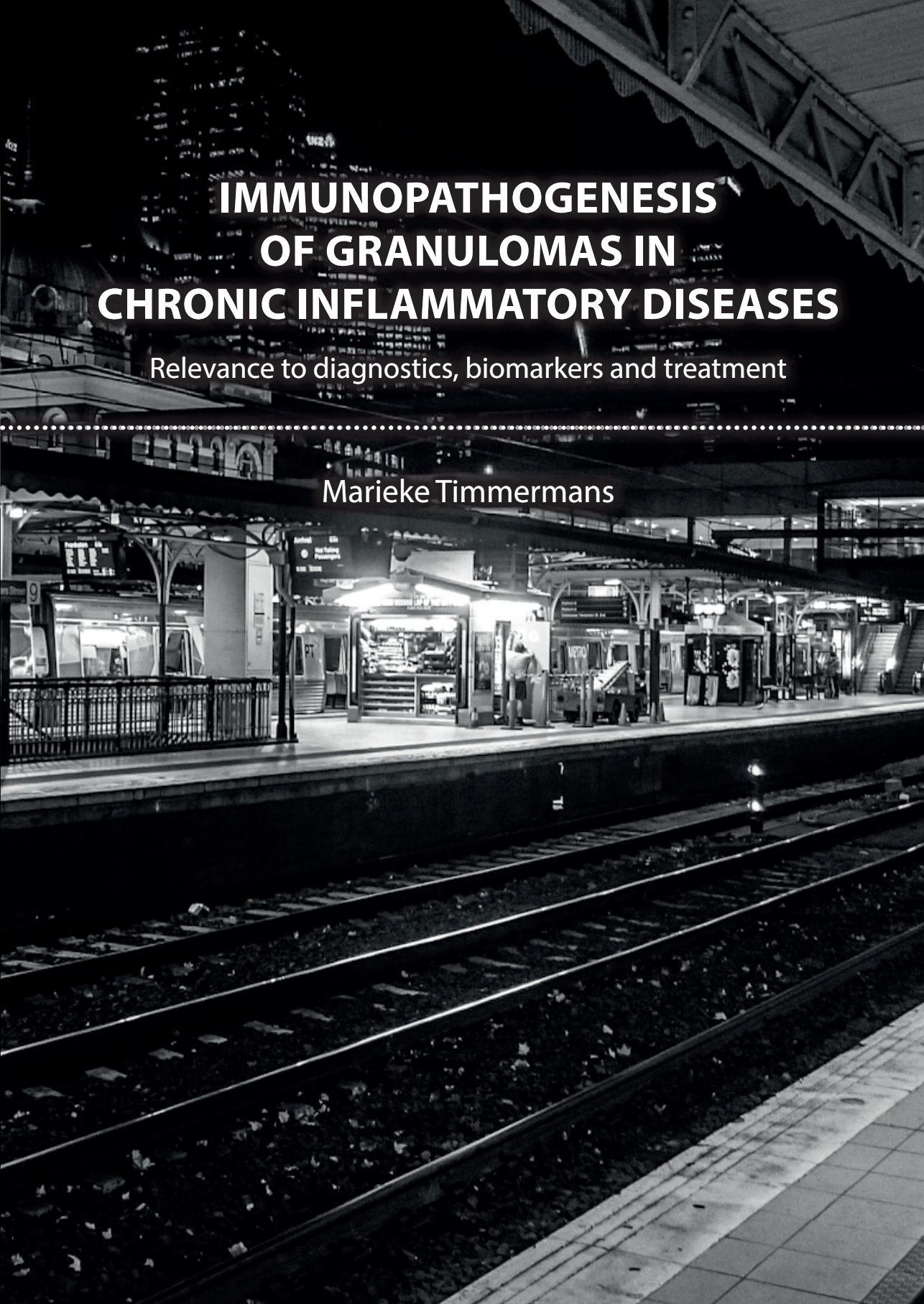


IMMUNOPATHOGENESIS OF GRANULOMAS IN CHRONIC INFLAMMATORY DISEASES

Relevance to diagnostics, biomarkers and treatment

Marieke Timmermans

IMMUNOPATHOGENESIS OF GRANULOMAS IN CHRONIC INFLAMMATORY DISEASES

Relevance to diagnostics, biomarkers and treatment

Marieke Timmermans

The research for this thesis was in part performed within the framework of the Erasmus Postgraduate School Molecular Medicine.

The studies described in the thesis were performed at the Department of Immunology, Department of Internal Medicine, Erasmus MC, University Medical Center Rotterdam, the Netherlands and Department Pathology and Immunology, Monash University, Melbourne, VIC, Australia.

The studies in this thesis were financially supported by Novartis, Coolsingel Foundation, Dutch Digestive Foundation and Dutch Association for Sarcoidosis Patients (Sarcoïdose Belangen Vereniging).

The printing of the thesis was supported by Elisabeth Tweesteden Ziekenhuis and Erasmus University.

ISBN

Cover Illustration	Anton Kerver
Layout	Nikki Vermeulen | Ridderprint BV
Printing	Ridderprint BV | www.ridderprint.nl

Immunopathogenesis of Granulomas in Chronic Inflammatory Diseases

Relevance to diagnostics, biomarkers and treatment

Immunopathogenese van granulomen in chronische ontstekingsziekten
Relevantie voor diagnostiek, biomarkers en therapie

Proefschrift

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus

Prof.dr. R.C.M.E. Engels

en volgens besluit van de College voor Promoties
De openbare verdediging zal plaatsvinden
op dinsdag 30 oktober 2018 om 9.30 uur

door

Wilhelmina Maria Cornelia Timmermans

geboren te Den Bosch

Erasmus University Rotterdam

PROMOTIECOMMISSIE

Promotor	Prof.dr. P.M. van Hagen
Overige leden	Prof.dr. L.J. Hofland Prof.dr. J.G.J.V. Aerts Prof.dr. D.L.P. Baeten
Copromotor	dr. J.A.M. van Laar dr. M.C. van Zelm

CONTENTS


Flinders Street
9

CHAPTER 1

GENERAL INTRODUCTION

Parts of this chapter were published in
Clinical and Translational Immunology. 2016 Dec 16;5(12):e118

ABSTRACT

Granulomas are clusters of immune cells. These structures can be formed in reaction to infection and display signs of necrosis, such as in tuberculosis. Alternatively, in several immune disorders, such as sarcoidosis, Crohn's disease and common variable immunodeficiency (CVID), non-caseating granulomas are formed without an obvious infectious trigger. Despite advances in our understanding of the human immune system, the pathogenesis underlying these non-caseating granulomas in chronic inflammatory diseases is still poorly understood. Here, we discuss the current knowledge about the immunopathogenesis of granulomas, and we discuss the different therapeutics.

INTRODUCTION

Inflammation is a physiological response of the body to invading pathogens. However, if the inflammatory state is not transient and persists chronically, this can result in irreversible tissue damage.[1] Typical non-infectious causes of chronic inflammation are autoimmune diseases, which are characterized by T-cell and antibody responses to self-antigens. Disorders that are characterized by innate immune responses without obvious auto-antibodies are referred to as autoinflammatory diseases.[2] In several autoinflammatory diseases, chronic inflammation can result in the formation of granulomas, which are clusters of immune cells in affected tissues.

The most common cause of all granuloma formation worldwide is tuberculosis.[3] The formation of granulomas in tuberculosis is thought to be a physiological reaction to prevent the systemic spread of the causative pathogen; the mycobacterium.[4] This immune response typically results in a caseating granuloma with signs of necrosis.[5] Many other infectious agents can trigger granuloma formation (Table 1.), as well as foreign body material such as beryllium, and inherited defects in neutrophil function (chronic granulomatous disease; CGD).[3,6-9] In chronic inflammatory diseases and primary immunodeficiencies with chronic inflammation, the granulomas have not been associated with specific external agents. With the exception of granulomatosis with polyangiitis (GPA), these granulomas are non-caseating and typically observed in patients with sarcoidosis,[10] Crohn's disease,[11] and common variable immunodeficiency (CVID)[12].

In recent years, several new insights have been generated into granulomatous inflammation. These new insights might soon be translated to clinical care, as increasing numbers of therapeutic agents targeting various immune pathways are currently tested in clinical trials.[13] Here, we review and discuss recent literature on granulomatous inflammation in sarcoidosis, Crohn's disease and CVID, all chronic inflammatory disorders with similar types of granulomas without a known trigger. We will specifically address the immune components involved in granuloma formation and how these can be utilized as disease markers and targeted by new therapeutic approaches for chronic autoinflammatory diseases with granuloma formation.

TABLE 1. Overview of infectious and non-infectious diseases with granuloma formation.

Category	Disease	Type of granuloma	Localization
Infectious			
Bacterium	tuberculosis	caseating necrosis	lung, extrapulmonary; disseminated
	brucellosis	necrotizing and fibrotic	liver, spleen
	bartonellosis	necrotizing	liver
	actinomycosis	non-caseating	cervicofacial, abdominal, lung
Fungus	histoplasmosis	necrotizing	lung
	aspergillosis	necrotizing	lung
	candidiasis	necrotizing with abcesses	skin
	cryptococcal disease	fibrotic with abcesses	lung
Parasitic	leishmaniasis	necrotizing	skin
	dirofilariasis	fibrotic and calcifying	subcutaneous
	schistomiasis	non-caseating	liver, intestines, bladder
Viral	CMV	unspecified	spleen and liver
	EBV	unspecified	skin
	measles	unspecified	thyroid gland
Non-infectious with known cause			
Primary Immunodeficiency	CGD	non-caseating	skin, intestines, liver
Malignancy	lymphoma	non-caseating	lymphatic tissue
	foreign body	non-caseating	tissue with contact to foreign body particle; skin, lung, intestines
Other	berylliosis	non-caseating	lung
Non-infectious with unknown cause			
Chronic inflammatory disease	GPA	necrotizing	lung, upper airways
	sarcoidosis	non-caseating	lung, skin, eye, lymph node, liver, CNS, heart
	Crohn's disease	non-caseating	intestines, skin, liver, lymph node
Primary Immunodeficiency	CVID	non-caseating	lung, lymph node, liver, skin, spleen, intestines

This Table is a non-exhaustive list of causes of granuloma formation. The affected organs are listed from the most commonly involved organ on the left to less common. The information is derived from references [3,6-9,14,15]. Abbreviations: CGD, chronic granulomatous disease; CMV, cytomegalovirus; CNS, central nervous system; CVID, common variable immunodeficiency; EBV, Epstein-Barr virus; GPA, granulomatosis with polyangiitis.

CHRONIC AUTOINFLAMMATORY DISEASES WITH GRANULOMA FORMATION

SARCOIDOSIS

Sarcoidosis is a multisystem granulomatous disease of unknown etiology. The hallmark of this disease is the presence of non-caseating granulomas affecting multiple organs. It is a rare disease with a worldwide prevalence ranging from 1 to 40 per 100,000 and a peak incidence at 20-39 years of age.[16] The clinical presentation of sarcoidosis is highly variable and dependent on the organs involved. Systemic complaints of fever, weight loss and fatigue are common. About 90% of patients have pulmonary granulomas with frequent involvement of other organs such as lymph nodes, skin, liver, eye, and heart.[10] A rare manifestation of sarcoidosis is the involvement of the nervous system in about 5% of cases.[17] Every aspect of the nervous system can be affected, however the central nervous system and the cranial nerves are most commonly affected.[18,19]Due to the high variability in clinical manifestations it can be challenging to diagnose sarcoidosis., especially in neurosarcoidosis, a rare and variable neurological manifestation. There is no definite test and diagnosis of sarcoidosis is based on three elements: 1) clinical and radiographic manifestations; 2) exclusion of diseases that may present similarly; 3) Identification of non-caseating granulomas by histological analysis of tissue.[20] Chest X-ray and computed tomography (CT) are the most common used visualization techniques. Radiographic pulmonary manifestations can vary from bihilar lymphadenopathy, pulmonary infiltration or fibrosis.[21] Nuclear techniques, such as the Fluorine-18 fluorodeoxyglucose positron emission tomography (18F-FDG PET), can also be used to evaluate extrapulmonary manifestations of sarcoidosis or to find a location for biopsy.[22] Blood tests can provide supportive information for making the diagnosis through detection of high serum levels of angiotensin converting enzyme (ACE) or soluble interleukin 2 receptor (sIL-2R), which is a marker for increased activation of T cells.[16,23]

Fortunately, treatment is not necessary in over 50% of patients in whom the disease will resolve in three years without medication.[10,16] Patients are only given medication when inflammation leads to organ damage. First-line therapy for sarcoidosis is based on corticosteroids such as prednisone. Second-line treatment comprises immunosuppressive medication such as methotrexate and azathioprine. For refractory cases, third line medication is available in the form of biologicals that block tumor necrosis factor (TNF)-α: infliximab or adalimumab.[24] This approach is successful in about 50% of treated patients in whom the granulomas resolve with no or little remaining organ damage. However, 20-25% of all diagnosed patients develop chronic disease with pulmonary fibrosis.[16] Current therapies target inflammatory pathways and have little effect on fibrosis. This is a major limitation because fibrosis results in increased

morbidity and mortality and the need for lung transplantation.[25] Patients with progressive neurological disease are often refractory to long term treatment with high dose corticosteroids and DMARD's or experience serious side effects. Third line treatment with infliximab appears to be effective in the majority of patients leading to a partial or complete remission. However, the two largest cohort studies on this subject both show a relapse rate of 50-56% after discontinuation of this TNF-blocker.[26,27]

The lack of a cure for sarcoidosis underlines the need to find new, effective drugs.[10,16]

CROHN'S DISEASE

Crohn's disease is an inflammatory bowel disease (IBD).[11] In recent years, the worldwide prevalence of Crohn's disease has been reported to increase, with current estimates in Western countries of 25 to 318 per 100,000.[28] Similar to sarcoidosis, Crohn's disease typically affects young adults, but with a 10-fold higher prevalence. The chronic inflammation in the intestinal tract is thought to result from an interplay of the genetic background, environmental factors, intestinal microbiota and a dysregulated immune system.[29] In Crohn's disease, chronic inflammation can manifest throughout the gastrointestinal tract, mainly affecting the ileum and the colon resulting in abdominal pain and diarrhea with passage of mucus or blood.[11] In addition, subsets of patients show inflammation of the skin, eyes or joints. Diagnosis of Crohn's disease is based on clinical assessment and physical examination of the patient in conjunction with imaging and histopathology of inflamed tissues and with blood tests.[11] Crohn's disease has many overlapping features with ulcerative colitis (UC),[30] the other major variant of IBD. In contrast to Crohn's disease, inflammation in UC is restricted to the colon and does not result in granuloma formation. When IBD is suspected, a colonoscopy is performed during which biopsies are taken. The histological finding of a non-caseating granuloma is the most discriminating factor for Crohn's disease.[31] Supporting evidence from laboratory analyses include high CRP, low Hb and high fecal calprotectin.[11] Furthermore, the majority of patients has detectable serum levels of anti-Saccharomyces cerevisiae antibodies (ASCA),[32] or antibodies to the outer membrane porin C of *E. coli* (anti-OmpC).[33] Despite granulomas being a discriminating factor with UC, these structures are only identified in about 37% of patients with Crohn's disease.[30] The presence of granulomas is associated with higher rates for surgical bowel resection, indicating that these are an indicator for severe disease.[30] Treatment of Crohn's disease is similar to sarcoidosis and includes corticosteroids, immunosuppressive and biologicals. In spite of the introduction of infliximab, treatment outcomes remain suboptimal with disease control being achieved in only 60% of Crohn's patients, [34] and intestinal complications and the requirement for surgery remain.[11]

CVID WITH GRANULOMATOUS COMPLICATIONS

CVID is a primary immunodeficiency (PID). It is a rare, heterogeneous disease with a prevalence of 2 to 4 per 100,000 and mean age of diagnosis between 30 and 40 years.[36] Patients suffer from recurrent sino-pulmonary infections and to a lesser extent from gastrointestinal infections. The hallmark of CVID is a B-cell defect leading to low or absent levels of immunoglobulins, and can be accompanied by abnormal T-cell responses and cytokine defects. Diagnosis of CVID is made when a patient has severely reduced levels of serum IgG with low IgM and/or IgA, and fulfills all of the following criteria: 1) onset after 2 years of age. 2) Poor or absent vaccination response. 3) Exclusion of other causes of hypogammaglobinemia.[37] Despite these commonalities in immunological defects and recurrent infections, CVID represents a heterogeneous group of patients with ranging clinical features that include autoimmunity, granuloma formation and hematological malignancies. These non-infectious complications are associated with high morbidity and early mortality.[38] Previously, only in 2-10% of patients a molecular cause of disease was identified in genes such as *ICOS, CD19, CD81, TNFRSF13C* (encodes BAFFR) and *TNFRSF13B* (encodes TACI).[39-43] However, none of these correlated with the incidence of granulomatous complications in 8-22% of CVID patients.[12] With the recent identification of autosomal dominant causes of complex antibody deficiencies and incomplete penetrance of some mutations (e.g. *CTLA4*, *PIK3CD*, *PIK3R1* and *NFKB1*),[44-47] it will become possible to relate granulomas to a genetic cause.

In CVID patients, granulomas most prevalently affect the lungs, followed by lymph nodes, liver, skin and spleen. The presence of granulomas can precede the diagnosis of CVID for years resulting in a potential misdiagnosis of sarcoidosis. However, sarcoidosis patients do not present with recurrent infections or low/absent immunoglobulins, because serum IgG levels are normal or even elevated in sarcoidosis.[48] CVID patients can also present with abdominal complaints, such as chronic diarrhea, weight loss and histological evidence of intestinal inflammation, resulting in an overlap of clinical features with Crohn's disease.[49] CVID patients with granulomas are more frequently affected by other autoimmune manifestations and have a higher morbidity and mortality rate than non-granulomatous patients.[12,50] The primary treatment of CVID is intravenous or subcutaneous immunoglobulin substitution, which is highly effective in reducing the infectious burden.[51] However, this treatment does not ameliorate the non-infectious complications. Conversely, granulomatous inflammation in CVID is treated with similar types of immune suppressive agents that are used for sarcoidosis and Crohn's disease. The combination of immunodeficiency with inflammation highlights the complicated processes involved in CVID, because it appears contrasting to treat immunocompromised patients with immunosuppressive medication.

While granulomas are the hallmark of disease in sarcoidosis, these are only detected in subgroups of patients with Crohn's disease and CVID. However, the exact incidence of granulomas in these disorders remains unclear and might be underestimated due

to sampling errors.[30] Furthermore, granulomas in CVID are often poorly recognized by physicians or upon discovery the patient is misdiagnosed with sarcoidosis.[12] As granulomatous complications are a predictor for poor disease outcome in CVID[12,50] and a pathognomic feature in Crohn's disease,[52] detection of these inflammatory structures is important in diagnostic work-up. As the finding of a granuloma as a varied differential diagnosis demonstrated in Table 1, it is important to look for distinguishing features such as necrosis. Nevertheless, diagnosis of all these diseases is based on the combination of clinical characteristics, pathology reports and other diagnostic tests.

KEY PLAYERS IN GRANULOMA PATHOGENESIS

ANTIGENIC TRIGGERS

Granulomas are thought to be formed following by a foreign trigger. Therefore, in diseases thus far characterized by non-infectious granulomatous inflammation, the search for a causative agent is still ongoing. In sarcoidosis there is a particular interest in finding the responsible trigger. An increased number of sarcoidosis cases was reported in rescuers after the terrorist attack on the World Trade Center in New York,[53] suggesting an external antigenic cause. Mycobacteria and Propionibacterium acnes are of specific interest, because DNA of these antigens was found in granuloma material from sarcoidosis patients with numbers ranging from 0-9 % for *M. tuberculosis* and 79-100% for *P. acnes*.[54] However, the causality of one single pathogen is debatable with such diverse pathogens being proposed.[10] Antigenic agents have also been suggested to trigger granuloma formation in Crohn's disease, mainly because of the associated defective bacterial clearance by autophagy. Polymorphisms in genes involved in autophagy have been reported,[55] the mechanism by which cells degrade and recycle of cellular components. In Crohn's disease this leads to the impaired capacity to handle pathogens by specialized intestinal epithelial cells, Paneth cells.[56] Furthermore, the presence of ASCA and anti-OmpC antibodies are suggestive of fungal or bacterial triggers of granuloma formation.[32,33] Finally, the high prevalence of *M. avium* in blood and tissue suggested that, similar to sarcoidosis, granulomas in Crohn's disease were formed in response to mycobacteria.[57,58] This theory is considered controversial, because *M. avium* is not typically pathogenic in humans and treatment of patients with anti-mycobacterial agents was proven ineffective.[59] An antigenic driver for persistence of granulomas in CVID is unlikely, because these patients are regularly treated with antibiotic or antifungal drugs, and these do not effectively resolve this type of inflammation.[12,60] Yet, a high prevalence of human herpesvirus type 8 (HHV8) is reported in granulomatous or lymphocytic interstitial lung disease (GLILD) patients (67%) as compared to the low prevalence of 4.8% in patients with CVID without GLILD. HHV8 infection might therefore

contribute to the poor prognosis of patients with granulomatous CVID.[61]

In conclusion, there is no unambiguous evidence for specific causal factors that trigger non-infectious granulomatous inflammation. It is evident that the immune system drives tissue destructive inflammation, but it remains to be determined if certain infectious or non-infectious particles are prone to trigger formation or persistence of granulomas.

MACROPHAGES

Macrophages are immune cells that are specialized in clearing of degraded extracellular substances through phagocytosis. These specialized immune cells are derived from circulating monocytes and are typically found in granulomas (Figure 2). Macrophages are thought to be one of the first cell types to migrate into affected tissue to clear debris and recruit other immune cells.[62] An important cytokine produced by macrophages is TNF-α, which induces vasodilation and thereby facilitates the infiltration of monocytes and lymphocytes. Macrophages also release other pro-inflammatory cytokines such as IL-1, IL-6, IL-12 and IL-23. Together with TNF-α, these cytokines promote leukocyte infiltration and T-cell activation, while inhibiting regulatory T cells (Treg) and T-cell apoptosis.[62] These activated macrophages are important in cell-mediated inflammation seen in granulomas, yet they also induce tissue damage. Polarization of macrophages mirrors the Th immune response status. Macrophages can acquire different functionalities in response to local triggers.[63] One definition to describe the activated state of macrophages is the classical M1 and alternative M2 activation. M1 macrophages are activated by Toll-like receptors and IFN-γ produced by Th1 cells.[64,65] M2 macrophages are activated through IL-4 and IL-13 and secrete extracellular matrix components promoting tissue remodeling.[64,65] Inflamed tissue in patients with Crohn's disease predominantly contain M1 macrophages,[66] and these contribute to the intestinal inflammation by disrupting the epithelial barrier in Crohn's disease.[67] A similar M1 polarization was seen in alveolar macrophages of patients with sarcoidosis.[68] Interestingly, an M2 polarization has been reported in other interstitial lung diseases with fibrosis. This is in line with an M2 polarization in a Th2 environment that has been observed in neurosarcoidosis with myofibrisosis,[69] and in fibrotic intestinal lesions of patients with Crohn's disease.[70] These studies suggest a M1 activation predominantly in the acute pro-inflammatory granulomatous inflammation with a possible shift towards M2 macrophages in fibrotic processes.

Stimulated macrophages can further mature into epithelioid cells that are elongated and resemble epithelial cells. Epithelioid cells appear to lose their phagocytic function and shift to more secretory capacities.[71,72] However, to our knowledge, it remains unclear what soluble factors these epithelioid cells produce. Epithelioid cells can fuse together and create compact aggregations, which are called multinucleated giant cells.[73] In

contrast to epithelioid cells, these multinucleated cells are capable of phagocytosis and cytokine secretion, esp. IL-1, TNF-α, and TGFβ.[74]

Our understanding of TNF-α and its role in granuloma integrity is mostly based on tuberculosis animal models.[75,76] In the absence of TNF, primary granulomas can still be formed. However, granulomas appeared disorganized.[75,76] Furthermore, a loss of TNF signaling disrupts already formed granulomas. This could in part be due to impaired lymphocyte recruitment and activation, in which TNF-α also plays a major role.[75]

Several abnormalities in monocyte and macrophage function have been reported in sarcoidosis, CVID and Crohn's disease, and these might contribute to the chronic inflammation and granuloma formation. Specifically, monocytes in patients with sarcoidosis and Crohn's disease have an increased ability to form multinucleated cells.[77,78] Furthermore, cultured alveolar macrophages of patients with sarcoidosis spontaneously produce more pro-inflammatory cytokines, including TNF-α, than controls,[79] and these higher levels were associated with progressive disease.[80] TNF-α production was also found to be increased in monocytes of patients with CVID.[81] The *TNF* 488A allele leads to higher TNF production and is strongly positively associated with granulomatous CVID.[82] Furthermore, 82% of *TNF* 488A allele negative patients were *IL-10* a-t-a allele positive, leading to lower IL-10 production resulting in a more pro-inflammatory TNF environment. Together, these two genetic variants seem to promote a cytokine shift contributing to an inflammatory environment leading to granulomatous complications.[83] The intestinal microbiota can also affect the inflammatory environment. Intestinal macrophages of patients with Crohn's disease produced more pro-inflammatory cytokines such as TNF-α after stimulation with commensal bacteria,[84] whereas reduced levels of pro-inflammatory cytokines were reported in response to *E. coli*.[85-87] Furthermore, *E.* coli is able to survive and replicate in intestinal macrophages in patients with Crohn's disease, is present in granulomas and can induce granuloma formation *in vitro*.[88-90] Due to this apparent decreased macrophage function, it has been proposed that Crohn's disease should also be considered a primary immunodeficiency.[91,92]

T CELLS

The inflammatory mediators produced by macrophages in affected tissue trigger the recruitment of additional immune cells, especially $CD4^+$ Helper T (Th) cells (Figures 1 and 2). Th cells are important mediators of immune responses and are thought to organize the granulomatous structure together with the already present macrophages. Traditionally, helper T cells were divided in Th1 and Th2 subsets, and the Th cells in granulomatous tissue were assumed to be type 1 cells that produce IL-2 to induce T-cell proliferation and the accumulation of effector T cells. However, with the more recent detection of other subsets such as Th17 cells and Tregs, the concepts of Th mediated inflammation have changed.[93]

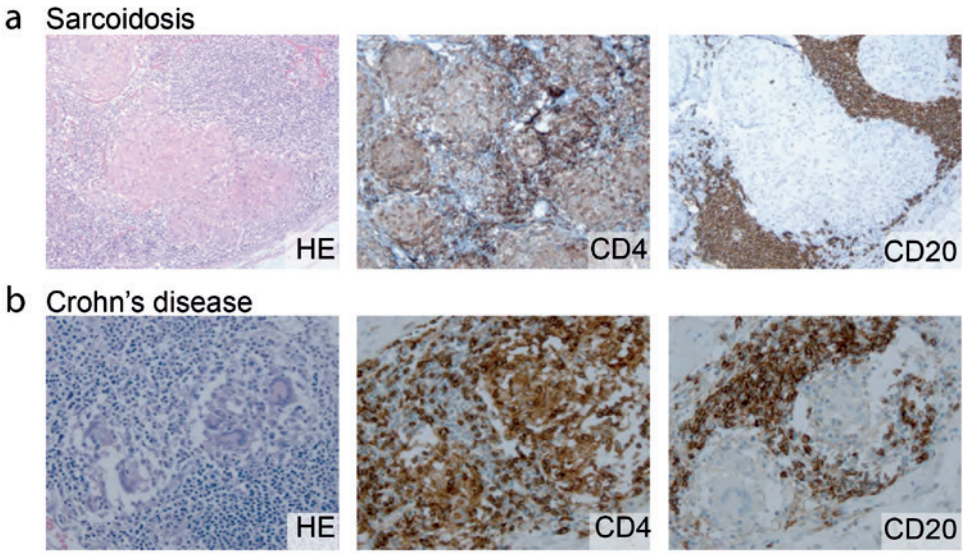


FIGURE 1. Non-caseating granulomas in Crohn's disease and sarcoidosis. Haemotoxylin and eosin stainings reveal granulomatous structures in a lymph node biopsy of a patient with sarcoidosis (**a**) and a biopsy of the ileum of a patient with Crohn's disease (**b**). Typically, CD4-expressing Th cells are detected in an around the granulomas, whereas CD20-expressing B cells are found to accumulate around the granulomas.[35]

Naive Th cells have the ability to differentiate in a particular subset through a specific cytokine milieu. The major subsets are Th1, Th2, Th17 and regulatory T cells (Treg) that are defined by their cytokine profiles and distinct effector functions (Figure 3). Th1 cells develop in the presence of IFN-γ and IL-12 and protect against intracellular pathogens through the production of IFN-γ and the resulting macrophage activation.[62,93] Upregulation of cytokines promoting Th1 differentiation have been reported in sarcoidosis: IL-2, IL-12, IL-15 and IL-18.[94] Th cell involvement in sarcoidosis is underpinned by the typical CD4 T-cell lymphopenia in peripheral blood in combination with CD4 T-cell infiltrates at the site of inflammation, such as in bronchoalveolar lavage fluid (BALF).[95,96] Despite these signs of local T-cell hyperactivity, the typical diminished cutaneous response to tuberculin is suggestive of T-cell anergy in non-granulomatous tissue.[97] CD4 T-cell anergy in these patients is likely due to chronic stimulation and results from reduced availability of G proteins,[98] and reduced NF-κB capacity of these cells.[99]

Patients with Crohn's disease show overexpression of IL-12 in intestinal tissue leading to increased production of IFN-γ.[100,101] Still, total blood CD4 T-cell numbers as normal, and even an expansion of CD4 memory T cells has been observed in patients with active Crohn's disease.[54] The hyperactive state of inflammation in Crohn's disease is further illustrated by mucosal T-cell proliferation and expansion with resistance to apoptosis.[102] Unlike sarcoidosis and Crohn's disease, patients with granulomatous CVID have low levels of total T cells and naive CD4 T cells.[82] This decrease could be related

to the immunodeficiency and result from increased T-cell turnover and apoptosis or decreased thymic output. It remains unclear whether this decrease also distinguishes granulomatous inflammation from Crohn's disease and sarcoidosis or it is the result of migration of T cells from circulation to the affected tissue.

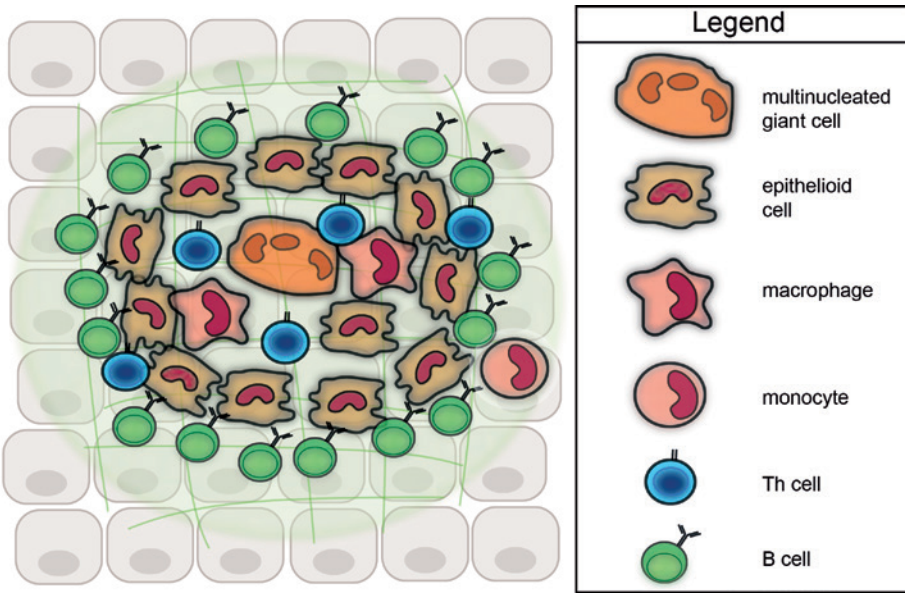


FIGURE 2. Model of the cellular organization of a non-caseating granuloma. Histology of granulomatous tissue (e.g. in Figure 1) display the presence of macrophages, epithelioid cells and multinucleated giant cells in the core of the granuloma. Th cells are localized in and around the granuloma. B cells are rarely seen in granulomatous structures, however they are numerously present around granulomas.[35]

In addition to Th1 responses, other Th subsets have been implicated in chronic inflammation. It is thought that the initial Th1 response during acute granulomatous inflammation shifts to a Th2 response in response when this becomes chronic. The production of Th2 cytokines can activate and stimulate fibroblasts and thereby contribute to fibrosis.[25]

More recently, Th17 have been shown to be disruptive in chronic inflammatory diseases.[103] Th17 cells are generated in the presence of IL-6 and TGF-β, and in turn produce IL-17 and IL-22 that are major factors in responses against extracellular pathogens and fungi (Figure 3). IL-17 was proposed to be a key mediator of inflammation in rheumatoid arthritis (RA), yet anti-IL-17 therapy with secukinumab was not effective.[104] Therefore, the exact role of Th17 cells in inflammatory disorders is not clear and information is mostly based on animal models. IL-17 overexpression leads to tissue damage in different organs such as lungs, intestines, joints and brain.[105] Th17 cells have the ability to change to a Th1 phenotype enabling cells to produce both IFN-γ and IL-17 referred to

as Th1/Th17 cells.[106] The plasticity of Th17 cells enables to further enhance inflammation either directly through the co-production of IL-17 and IFN-γ, or through providing help in generation of new pathogenic Th1 cells.[107] Moreover, Th17 cells in mouse models have recently been shown to adapt into a regulatory phenotype with a change in transcriptional profile and regulatory capacities.[108]

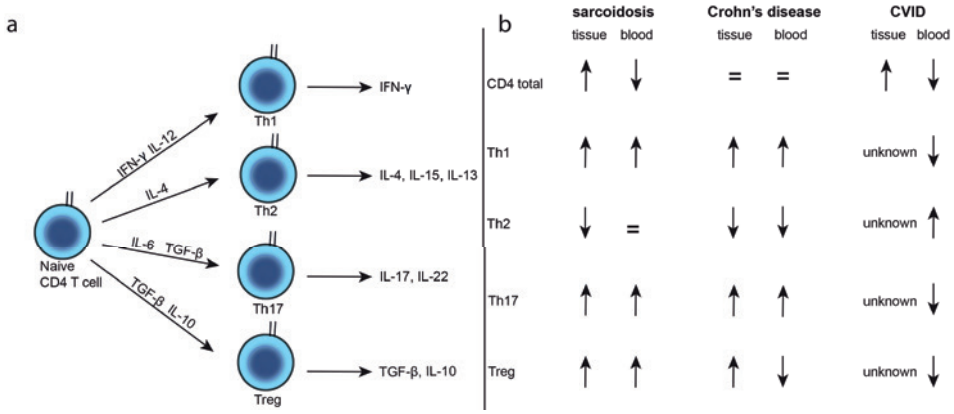


FIGURE 3. Involvement of CD4+ Th-cell subsets in three chronic granulomatous inflammatory diseases. (**a**) Model of Th cell maturation into Th1, Th2, Th17 and Treg subsets. Key cytokines are depicted. (**b**) Summary of observations on total CD4+ Th as well as Th1, Th2, Th17 and Treg subsets in tissue and blood of patients with sarcoidosis, Crohn's disease and CVID.[95,96,119,120,125-132]

In both sarcoidosis and Crohn's disease IL-17 expression is increased in inflammatory tissue, concomitant with an increase of Th17 cells in the peripheral blood.[29,109] In contrast, CVID patients have low Th17 cell numbers in their peripheral blood, which is associated with higher numbers of $CD21^{low}$ B cells and lower numbers of memory B cells.[110] The presence of an expanded $CD21^{low}$ B-cell population in CVID patients is associated with higher incidence of non-infectious complications.[111] The concomitant decrease in Th17 cells is suggestive of a combined defect in B- and T-cells in this subset of CVID patients. The nature of this defect remains to be determined and could be B- or T-cell intrinsic or arise from impaired regulation of Th maturation.[110]

Regulatory T cells (Tregs) are important to dampen immune responses and thereby maintain a physiological immune homeostasis and self-tolerance.[112] Naive T cells can mature into Tregs through expression of the transcription factor Forkhead box p3 (FoxP3) in the context of TGF-β, subsequently exerting immune regulatory functions through production of TGF-β and IL-10.[93] Tregs became an intensively studied cell-population after it was reported that $CD4^{+}CD25^{+}$ depletion in mice resulted in a variety of autoimmunity including gastrointestinal involvement.[113] Furthermore, patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), a genetic disorder caused by mutation in the *FOXP3*_gene, are affected by excessive gastrointestinal autoimmunity.[114]

In patients with sarcoidosis, higher frequencies of Tregs have been reported in both peripheral blood and BALF with accumulation of Tregs in the vicinity of granulomas.[115] These Tregs inhibited T-cell proliferation, yet several groups confirmed a decreased suppressor function on $CD4^+$ cells.[115-117] Moreover, Tregs from patients with active sarcoidosis were not able to suppress granuloma formation in an *in vitro* model, while Tregs cells from healthy controls were.[118] However, it remains unclear whether Tregs were defective or were merely exhausted as a result of the continuous inflammation. Patients with active Crohn's disease have decreased Treg numbers in blood, whereas these are increased in the intestinal mucosa.[119,120] The anti-inflammatory function of Tregs is likely to be intact as they have preserved suppressor function,[120] and are able to inhibit effector T-cell responses.[119] However, it has been postulated that effector T cells in the lamina propria are unresponsive to the inhibiting effects of Tregs, implicating a contributing factor to the chronic inflammatory response.[119] Treg function in CVID has been less well documented, yet decreased levels of Tregs in blood of CVID patients were specifically seen in patients with autoimmune complications.[121] Importantly, Tregs require the inhibitory receptor cytotoxic T-lymphocyte-antigen 4 (CTLA-4) for suppressive function, and mutations in *CTLA4* underlie an immunodeficiency in which Tregs have reduced suppressive function. These patients often present with granulomatous inflammation and intestinal inflammation with similarities to Crohn's disease.[47,122,123] As decreased CTLA-4 expression on Tregs has also been reported in patients with sarcoidosis, it is possible that defects in CTLA-4 and Treg function contribute to granuloma formation in autoinflammatory diseases.[124]

B CELLS

The main focus in granulomatous inflammation has previously been directed to macrophage and T-cell dysfunction. However, in addition to macrophages and T cells, B-cell infiltrates are present in granulomatous tissue of patients with tuberculosis.[133] Furthermore, several studies showed that numerous B cells surround granulomas in affected tissues from patients with sarcoidosis as well as Crohn's disease (Figure 1).[35,134,135] These B cells are likely to be essential for the development of granulomas as indicated by two findings: First, patients with CVID can develop granulomas, whereas patients with X-linked agammaglobulinemia (XLA) do not.[136] CVID and XLA patients both have an antibody deficiency due to B-cell dysfunction, but mature B cells are completely absent in XLA.[137] Second, in a mouse model of oil granulomas the absence of T cells did not affect the ability of granuloma formation, whereas granulomas were not formed in the absence of B cells.[138] Traditionally, B cells are regarded the antibody producing cells of the immune system. While this is a major function of B cells, it has become clear that B-cell development is a complicated process with many B-cell subsets and functions involved. Other B-cell functions include the ability to act as antigen presenting cells,

co-stimulate T cells, have regulatory effects and produce cytokines that direct Th subset maturation.[139] These insights, together with novel possibilities to target B cells with biologicals, provide a strong rationale to investigate the role of B cells in granulomatous inflammation.

Some recent insights have been generated into B-cell abnormalities in sarcoidosis and Crohn's disease. Patients with sarcoidosis carry reduced numbers of IgM, IgG, and IgA memory B cells and plasma cells in blood with the exception of $CD27^{-}IgA^{+}$ memory B cells.[35,140,141] Despite their reduced numbers, levels of somatic hypermutations in immunoglobulin (Ig) gene transcripts of these cells were increased, suggestive of chronic activation.[35] This is potentially related to serum B-cell activating factor (BAFF), a critical factor for mature B-cell survival of which the levels are increased in sarcoidosis patients with active disease.[140,141] Furthermore, levels of NF-κB transcription factors in B cells are reduced and potentially affect B-cell responses and proliferation.[142] How these B-cell abnormalities affect the formation of persistence of granulomas still needs to be determined, yet combined these result do suggest a disturbed B-cell homeostasis.

Similar to sarcoidosis, patients with Crohn's disease also display reductions in blood IgM memory B-cell numbers.[143] However, the B-cell compartment in Crohn's disease has not been as extensively studied as in sarcoidosis and CVID. CVID is characterized by hypogammaglobinemia and all patients have reduced blood plasma cells, which in many patients is accompanied by memory B-cell defects.[111] Furthermore, in subgroups of patients, expansions of transitional B cells, as well as $CD21^{low}$ B cells have been identified. Many of these abnormalities have formed the basis of flow cytometry-based classifications.[111] However, B-cell phenotypes do not seem to correlate well with severity of disease or non-infectious complications. Yet, granulomatous complications are found to be associated with lower numbers of Ig switched memory B cells.[60,111] Still, it remains unclear if these reductions are related to the immunodeficiency or the result of migration towards the sites of granulomatous inflammation. Patients with mutations in *ICOS* and TACI gene can develop granulomatous complications and autoimmunity in general.[144] These genes are involved in different pathways of B-cell survival and T cell-dependent or -independent antibody responses. With the implementation of whole exome sequencing, the genetics of CVID unravels rapidly. Possibly, this will provide better insights into affected processes and will help to dissect the mechanisms that, when impaired, result in granuloma formation.

Although it remains unclear how B cells contribute to disease pathogenesis, the common signs of chronic activation of B cells in granulomatous autoinflammatory diseases is suggestive of their role in ongoing inflammation. The systemic B-cell abnormalities could provide good markers for disease and treatment monitoring. Moreover, disease-specific abnormalities could provide more insight into pathogenesis and starting points for novel therapeutic approaches.

THERAPEUTIC IMPLICATIONS

REMISSION OR FIBROSIS?

In many patients granulomas persist and lead to organ damage due to fibrosis. Fibrosis is therefore a common problem in sarcoidosis and Crohn's disease,[16 145] The impact of granulomas on permanent organ damage in CVID patients is currently unknown due to the complications of recurrent respiratory infections that lead to bronchiectasis in 23% of patients.[36] Despite fibrosis leading to increased morbidity and mortality,[25] to date, therapies targeting inflammatory pathways do not resolve or delay the process. Moreover, it is not yet possible to identify which patients will develop fibrotic complications.[10] Therefore, exploring fibrotic pathways may lead to new and much needed therapies to prevent irreversible organ damage.

MECHANISM OF CURRENT THERAPIES

First and second line medication to treat patients with chronic inflammatory disease are corticosteroids and immunosuppressives such as methotrexate and azathioprine. Corticosteroids have anti-inflammatory properties though inhibition of leukocyte migration and pro-inflammatory cytokine production (esp. TNF-α and IFN-γ).[146] Methotrexate inhibits the purine metabolism and azathioprine purine synthesis, which both lead to decreased lymphocyte proliferation and cytokine release.[147] While these therapies are administered to suppress pro-inflammatory cytokines through inhibiting T-cell responses, these immunosuppressive drugs also affect the B-cell compartment.[148,149]

With the introduction of biological therapies, a third line of treatment has become available, of which TNFα-blockers are most notable. The most widely used TNFα-blockers are antibodies against TNFα (infliximab and adalimumab), which have proven to be effective in Crohn's disease and sarcoidosis (Figure 4).[150,151] This treatment specifically disrupts the granuloma structure. As this can result in reactivation of latent tuberculosis, all patients need to be intensively screened for tuberculosis prior to treatment with TNFα-blockers.[152] TNF-blockers infliximab and etanercept have proven to be beneficial in some patients with granulomatous CVID.[60,153] Etanercept is a recombinant TNFα receptor fused to an Ig constant region and is often used to treat RA.[154] Importantly, etanercept is not effective in sarcoidosis and Crohn's disease, and can even lead to increased disease activity in these disorders.[155,156] This might be related to its different biological properties as opposed to anti-TNF antibodies: 1) etanercept binds only to soluble trimeric and not monomeric soluble TNF-α; 2) Etanercept has low affinity to transmembrane TNF;[157] 3) Etanercept binds to both TNF-α and lymphotoxin alpha (LTα), a cytokine that is crucial for secondary lymphoid organ development, IgA regulation and

T-cell gut homing.[158] These abilities could explain the reduced effectivity of etanercept in Crohn's disease and sarcoidosis, as well as observed disease complications. Treatment with TNFα-blockers also affects the blood B-cell compartment in patients with Crohn's disease and sarcoidosis.[145,159] It remains to be determined if this is an indirect effect following modulation of inflammation or if this is through direct binding to TNFRII that is expressed on B cells.

Targeting of T cells in granulomatous diseases has yielded mixed results. A clinical trial for treatment of patients with Crohn's disease with abatacept was ineffective.[160] Abatacept is a recombinant fusion protein of CTLA4 with an immunoglobulin. CTLA-4 inhibits T-cell activation by binding to CD28 on T cells. Abatacept has shown beneficial effects in RA patients,[161] and in animal models of intestinal inflammation. These results illustrate that, in spite of unravelling underlying immune mechanisms, translation into effective therapies for human autoinflammatory disease remains challenging.

Targeting of Th17 responses have also been studied. However, blocking IL-17 with secukinumab was ineffective in patients with Crohn's disease,[162] whereas treatment with brodalumab an anti-IL-17 receptor monoclonal antibody, even resulted in exacerbation of Crohn's disease.[163]

Ustekinumab, a monoclonal antibody against both IL-12 and IL-23, resulted in a clinical response in patients with refractory Crohn's disease[164] and is currently implemented in patients who are resistant to TNFα-blockers.[165] However, ustekinumab did not show therapeutic efficacy in sarcoidosis patients.[166]

Patients with Crohn's disease do show a good response to treatment with vedolizumab, a humanized monoclonal antibody that binds to integrin $\alpha_4\beta_7$.[167] As $\alpha_4\beta_7$ specifically mediates gut homing, it can selectively inhibit intestinal inflammation. Because granulomas in patients with sarcoidosis and CVID more frequently present in other tissues than the gut, vedolizumab is likely to have limited effects in these diseases. Targeting of B cells with rituximab has shown promising results in granulomatous CVID.[60] Rituximab is a humanized anti-CD20 antibody that depletes all naive and memory B cells.[168] The efficacy of rituximab in sarcoidosis is still unclear: several case reports show proven effectivity, however, one small prospective study with 10 patients only 5 of them showed a marginal (>5%) improvement of respiratory function.[169] In contrast, a patient with Crohn's disease displayed disease exacerbation following treatment with rituximab, implying a protective role for B cells in Crohn's disease.[170] These different outcomes of rituximab treatment highlight the complexity of the underlying inflammatory processes.

AIMS AND OUTLINE OF THIS THESIS

Granulomatous inflammation consists of a complex interplay between macrophages, different types of T cells and B cells. Granulomas are the hallmark of sarcoidosis, but are also present in a subgroup of patients with Crohn's disease. The immunopathophysiology of granulomatous inflammation is the backbone of this thesis. By unraveling the different components in tissue and blood we aim to eventually translate the bench into bedside to improve diagnostics, identify biomarkers for therapy and search for new therapeutic targets in these chronic inflammatory diseases. In **Chapter 2** the hypothesis is that B cells are involved in (granulomatous) inflammation in Crohn's disease. Therefore, we study whether B cells are, in accordance with sarcoid granulomas, present in the intestinal tissue of patients with Crohn's disease, and if patients have systemic abnormalities in their blood B-cell compartment. In **Chapter 3**, the presence of B cells around granulomas is utilized to study if immunohistochemical stainings of biopsies for B cells can be used as a diagnostic tool to increase the sensitivity for detection of granulomas and improve differential diagnosis between ulcerative colitis and Crohn's disease. **Chapter 4** continues to dissect the role of B cells in sarcoidosis and its possible use as biomarkers for treatment outcome. Therefore, we analyzed the effect of successful infliximab therapy on blood B –and T-cell subsets, serum immune markers and infliximab trough levels in patients with sarcoidosis aiming to find biomarkers for successful outcome of therapy. In **Chapter 5** we aim to elucidate the effect of infliximab on patients with neurosarcoidosis, a rare form of sarcoidosis which can be difficult to treat. As evidence in literature is limited to small case reports, we describe a multicenter cohort study of patients with neurosarcoidosis treated with infliximab. **Chapter 6** is the Discussion of this thesis and comprises the research results of these studies and compares results from both sarcoidosis and Crohn's disease. Furthermore, it shows results from a small trial of patients with chronic sarcoidosis treated with sandostatin therapy. Eventually, future therapeutics will be discussed.

REFERENCES

1 Medzhitov, R. Origin and physiological roles of inflammation. *Nature* **454**, 428-435, doi:10.1038/nature07201 (2008).

2 Doria, A. *et al.* Autoinflammation and autoimmunity: bridging the divide. *Autoimmunity reviews* **12**, 22-30, doi:10.1016/j.autrev.2012.07.018 (2012).

3 Mukhopadhyay, S. *et al.* Causes of pulmonary granulomas: a retrospective study of 500 cases from seven countries. *Journal of clinical pathology* **65**, 51-57, doi:10.1136/jclinpath-2011-200336 (2012).

4 Williams, G. T. & Williams, W. J. Granulomatous inflammation--a review. *Journal of clinical pathology* **36**, 723-733 (1983).

5 Orme, I. M. & Basaraba, R. J. The formation of the granuloma in tuberculosis infection. *Semin Immunol* **26**, 601-609, doi:S1044-5323(14)00091-8 [pii] 10.1016/j.smim.2014.09.009 (2014).

6 Woodard, B. H., Rosenberg, S. I., Farnham, R. & Adams, D. O. Incidence and nature of primary granulomatous inflammation in surgically removed material. *The American journal of surgical pathology* **6**, 119-129 (1982).

7 Levine, S., Smith, V. V., Malone, M. & Sebire, N. J. Histopathological features of chronic granulomatous disease (CGD) in childhood. *Histopathology* **47**, 508-516, doi:10.1111/j.1365-2559.2005.02258.x (2005).

8 Rossman, M. D. Chronic beryllium disease: a hypersensitivity disorder. *Applied occupational and environmental hygiene* **16**, 615-618, doi:10.1080/10473220121477 (2001).

9 Mukhopadhyay, S. & Gal, A. A. Granulomatous lung disease: an approach to the differential diagnosis. *Archives of pathology & laboratory medicine* **134**, 667-690, doi:10.1043/1543-2165-134.5.667 (2010).

10 Valeyre, D. *et al.* Sarcoidosis. *Lancet* **383**, 1155-1167, doi:10.1016/S0140-6736(13)60680-7 (2014).

11 Baumgart, D. C. & Sandborn, W. J. Crohn's disease. *Lancet* **380**, 1590-1605, doi:S0140-6736(12)60026-9 [pii] 10.1016/S0140-6736(12)60026-9 (2012).

12 Ardeniz, O. & Cunningham-Rundles, C. Granulomatous disease in common variable immunodeficiency. *Clin Immunol* **133**, 198-207, doi:S1521-6616(09)00661-5 [pii] 10.1016/j.clim.2009.05.001 (2009).

13 Tas, S. W. & Baeten, D. L. Recent Advances in the Treatment of Immune-Mediated Inflammatory Diseases. *Methods in molecular biology* **1371**, 143-155, doi:10.1007/978-1-4939-3139-2_9 (2016).

14 Valour, F. *et al.* Actinomycosis: etiology, clinical features, diagnosis, treatment, and management. *Infection and drug resistance* **7**, 183-197, doi:10.2147/IDR.S39601 (2014).

15 Petersen, H. J. & Smith, A. M. The role of the innate immune system in granulomatous disorders. *Front Immunol* **4**, 120, doi:10.3389/fimmu.2013.00120 (2013).

16 Iannuzzi, M. C., Rybicki, B. A. & Teirstein, A. S. Sarcoidosis. *The New England journal of medicine* **357**, 2153-2165, doi:10.1056/NEJMra071714 (2007).

17 Zajicek, J. P. *et al.* Central nervous system sarcoidosis--diagnosis and management. *QJM* **92**, 103-117 (1999).

18 Chapelon, C. *et al.* Neurosarcoidosis: signs, course and treatment in 35 confirmed cases. *Medicine (Baltimore)* **69**, 261-276 (1990).

19 Gascon-Bayarri, J. *et al.* Neurosarcoidosis: report of 30 cases and a literature survey. *Eur J Intern Med* **22**, e125-132, doi:S0953-6205(11)00192-0 [pii] 10.1016/j.ejim.2011.08.019 (2011).

20 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. *American journal of respiratory and critical care medicine* **160**, 736-755, doi:10.1164/ajrccm.160.2.ats4-99 (1999).

21 Scadding, J. G. Prognosis of intrathoracic sarcoidosis in England. A review of 136 cases after five years' observation. *British medical journal* **2**, 1165-1172 (1961).

22 Treglia, G., Taralli, S. & Giordano, A. Emerging role of whole-body 18F-fluorodeoxyglucose positron emission tomography as a marker of disease activity in patients with sarcoidosis: a systematic review. *Sarcoidosis, vasculitis, and diffuse lung diseases : official journal of WASOG / World Association of Sarcoidosis and Other Granulomatous Disorders* **28**, 87-94 (2011).

23 Grutters, J. C. *et al.* Serum soluble interleukin-2 receptor measurement in patients with sarcoidosis: a clinical evaluation. *Chest* **124**, 186-195 (2003).

24 Drent, M., Cremers, J. P., Jansen, T. L. & Baughman, R. P. Practical eminence and experience-based recommendations for use of TNF-alpha inhibitors in sarcoidosis. *Sarcoidosis, vasculitis, and diffuse lung diseases : official journal of WASOG / World Association of Sarcoidosis and Other Granulomatous Disorders* **31**, 91-107 (2014).

25 Patterson, K. C., Hogarth, K., Husain, A. N., Sperling, A. I. & Niewold, T. B. The clinical and immunologic features of pulmonary fibrosis in sarcoidosis. *Translational research : the journal of laboratory and clinical medicine* **160**, 321-331, doi:10.1016/j.trsl.2012.03.005 (2012).

26 Cohen Aubart, F. *et al.* Long-term outcomes of refractory neurosarcoidosis treated with infliximab. *J Neurol* **264**, 891-897, doi:10.1007/s00415-017-8444-9 10.1007/s00415-017-8444-9 [pii] (2017).

27 Gelfand, J. M. *et al.* Infliximab for the treatment of CNS sarcoidosis: A multi-institutional series. *Neurology* **89**, 2092-2100, doi:WNL.0000000000004644 [pii] 10.1212/WNL.0000000000004644 (2017).

28 Burisch, J. & Munkholm, P. The epidemiology of inflammatory bowel disease. *Scandinavian journal of gastroenterology* **50**, 942-951, doi:10.3109/00365521.2015.1014407 (2015).

29 Geremia, A., Biancheri, P., Allan, P., Corazza, G. R. & Di Sabatino, A. Innate and adaptive immunity in inflammatory bowel disease. *Autoimmunity reviews* **13**, 3-10, doi:10.1016/j.autrev.2013.06.004 (2014).

30 Heresbach, D. *et al.* Frequency and significance of granulomas in a cohort of incident cases of Crohn's disease. *Gut* **54**, 215-222, doi:10.1136/gut.2004.041715 (2005).

31 Feakins, R. M. Ulcerative colitis or Crohn's disease? Pitfalls and problems. *Histopathology* **64**, 317-335, doi:10.1111/his.12263 (2014).

32 Sendid, B. *et al.* Specific antibody response to oligomannosidic epitopes in Crohn's disease. *Clin Diagn Lab Immunol* **3**, 219-226 (1996).

33 Mei, L. *et al.* Familial expression of anti-Escherichia coli outer membrane porin C in relatives of patients with Crohn's disease. *Gastroenterology* **130**, 1078-1085, doi:S0016-5085(06)00275-7 [pii] 10.1053/j.gastro.2006.02.013 (2006).

34 Colombel, J. F. *et al.* Infliximab, azathioprine, or combination therapy for Crohn's disease. *The New England journal of medicine* **362**, 1383-1395, doi:10.1056/NEJMoa0904492 (2010).

35 Kamphuis, L. S. *et al.* Perigranuloma localization and abnormal maturation of B cells: emerging key players in sarcoidosis? *American journal of respiratory and critical care medicine* **187**, 406-416, doi:10.1164/rccm.201206-1024OC (2013).

36 Gathmann, B. *et al.* Clinical picture and treatment of 2212 patients with common variable immunodeficiency. *The Journal of allergy and clinical immunology* **134**, 116-126, doi:10.1016/j.jaci.2013.12.1077 (2014).

37 Conley, M. E., Notarangelo, L. D. & Etzioni, A. Diagnostic criteria for primary immunodeficiencies. Representing PAGID (Pan-American Group for Immunodeficiency) and ESID (European Society for Immunodeficiencies). *Clin Immunol* **93**, 190-197, doi:10.1006/clim.1999.4799 (1999).

38 Resnick, E. S., Moshier, E. L., Godbold, J. H. & Cunningham-Rundles, C. Morbidity and mortality in common variable immune deficiency over 4 decades. *Blood* **119**, 1650-1657, doi:10.1182/blood-2011-09-377945 (2012).

39 Grimbacher, B. *et al.* Homozygous loss of ICOS is associated with adult-onset common variable immunodeficiency. *Nature immunology* **4**, 261-268, doi:10.1038/ni902 (2003).

40 Pan-Hammarstrom, Q. *et al.* Reexamining the role of TACI coding variants in common variable immunodeficiency and selective IgA deficiency. *Nature genetics* **39**, 429-430, doi:10.1038/ng0407-429 (2007).

41 van Zelm, M. C. *et al.* An antibody-deficiency syndrome due to mutations in the CD19 gene. *The New England journal of medicine* **354**, 1901-1912, doi:354/18/1901 [pii] 10.1056/NEJMoa051568 (2006).

42 van Zelm, M. C. *et al.* CD81 gene defect in humans disrupts CD19 complex formation and leads to antibody deficiency. *J Clin Invest* **120**, 1265-1274, doi:39748 [pii] 10.1172/JCI39748 (2010).

43 Warnatz, K. *et al.* B-cell activating factor receptor deficiency is associated with an adult-onset antibody deficiency syndrome in humans. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 13945-13950, doi:10.1073/pnas.0903543106 (2009).

44 Angulo, I. *et al.* Phosphoinositide 3-kinase delta gene mutation predisposes to respiratory infection and airway damage. *Science* **342**, 866-871, doi:10.1126/science.1243292 (2013).

45 Deau, M. C. *et al.* A human immunodeficiency caused by mutations in the PIK3R1 gene. *J Clin Invest* **124**, 3923-3928, doi:10.1172/JCI75746 (2014).

46 Fliegauf, M. *et al.* Haploinsufficiency of the NF-kappaB1 Subunit p50 in Common Variable Immunodeficiency. *American journal of human genetics* **97**, 389-403, doi:10.1016/j.ajhg.2015.07.008 (2015).

47 Kuehn, H. S. *et al.* Immune dysregulation in human subjects with heterozygous germline mutations in CTLA4. *Science* **345**, 1623-1627, doi:10.1126/science.1255904 (2014).

48 Bouvry, D. *et al.* Granulomatosis-associated common variable immunodeficiency disorder: a case-control study versus sarcoidosis. *Eur Respir J* **41**, 115-122, doi:09031936.00189011 [pii] 10.1183/09031936.00189011 (2013).

49 Mannon, P. J. *et al.* Excess IL-12 but not IL-23 accompanies the inflammatory bowel disease associated with common variable immunodeficiency. *Gastroenterology* **131**, 748-756, doi:10.1053/j.gastro.2006.06.022 (2006).

50 Bates, C. A. *et al.* Granulomatous-lymphocytic lung disease shortens survival in common variable immunodeficiency. *The Journal of allergy and clinical immunology* **114**, 415-421, doi:10.1016/j.jaci.2004.05.057 (2004).

51 Salzer, U., Warnatz, K. & Peter, H. H. Common variable immunodeficiency: an update. *Arthritis research & therapy* **14**, 223, doi:10.1186/ar4032 (2012).

52 Turner, K., Genta, R. M., Lujan, G., Robiou, C. & Sonnenberg, A. Significance of the epithelioid granuloma in biopsies of Crohn's colitis. *Inflammatory bowel diseases* **20**, 2271-2275, doi:10.1097/MIB.0000000000000196 (2014).

53 Crowley, L. E. *et al.* "Sarcoid like" granulomatous pulmonary disease in World Trade Center disaster responders. *American journal of industrial medicine* **54**, 175-184, doi:10.1002/ajim.20924 (2011).

54 Eishi, Y. *et al.* Quantitative analysis of mycobacterial and propionibacterial DNA in lymph nodes of Japanese and European patients with sarcoidosis. *Journal of clinical microbiology* **40**, 198-204 (2002).

55 Rioux, J. D. *et al.* Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. *Nature genetics* **39**, 596-604, doi:10.1038/ng2032 (2007).

56 Thachil, E. *et al.* Abnormal activation of autophagy-induced crinophagy in Paneth cells from patients with Crohn's disease. *Gastroenterology* **142**, 1097-1099 e1094, doi:S0016-5085(12)00151-5 [pii] 10.1053/j.gastro.2012.01.031 (2012).

57 Naser, S. A., Ghobrial, G., Romero, C. & Valentine, J. F. Culture of Mycobacterium avium subspecies paratuberculosis from the blood of patients with Crohn's disease. *Lancet* **364**, 1039-1044, doi:10.1016/S0140-6736(04)17058-X S014067360417058X [pii] (2004).

58 Bull, T. J. *et al.* Detection and verification of Mycobacterium avium subsp. paratuberculosis in fresh ileocolonic mucosal biopsy specimens from individuals with and without Crohn's disease. *Journal of clinical microbiology* **41**, 2915-2923 (2003).

59 Mendoza, J. L., Lana, R. & Diaz-Rubio, M. Mycobacterium avium subspecies paratuberculosis and its relationship with Crohn's disease. *World J Gastroenterol* **15**, 417-422 (2009).

60 Boursiquot, J. N. *et al.* Granulomatous disease in CVID: retrospective analysis of clinical characteristics and treatment efficacy in a cohort of 59 patients. *J Clin Immunol* **33**, 84-95, doi:10.1007/s10875-012-9778-9 (2013).

61 Wheat, W. H. *et al.* Possible role of human herpesvirus 8 in the lymphoproliferative disorders in common variable immunodeficiency. *J Exp Med* **202**, 479-484, doi:jem.20050381 [pii] 10.1084/jem.20050381 (2005).

62 Arango Duque, G. & Descoteaux, A. Macrophage cytokines: involvement in immunity and infectious diseases. *Front Immunol* **5**, 491, doi:10.3389/fimmu.2014.00491 (2014).

63 Murray, P. J. *et al.* Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity* **41**, 14-20, doi:S1074-7613(14)00228-3 [pii] 10.1016/j.immuni.2014.06.008 (2014).

64 Wynn, T. A., Chawla, A. & Pollard, J. W. Macrophage biology in development, homeostasis and disease. *Nature* **496**, 445-455, doi:nature12034 [pii] 10.1038/nature12034 (2013).

65 Mosser, D. M. & Edwards, J. P. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* **8**, 958-969, doi:nri2448 [pii] 10.1038/nri2448 (2008).

66 Barros, M. H., Hauck, F., Dreyer, J. H., Kempkes, B. & Niedobitek, G. Macrophage polarisation: an immunohistochemical approach for identifying M1 and M2 macrophages. *PLoS One* **8**, e80908, doi:10.1371/journal.pone.0080908 PONE-D-13-29825 [pii] (2013).

67 Lissner, D. *et al.* Monocyte and M1 Macrophage-induced Barrier Defect Contributes to Chronic Intestinal Inflammation in IBD. *Inflammatory bowel diseases* **21**, 1297-1305, doi:10.1097/MIB.0000000000000384 (2015).

68 Wojtan, P., Mierzejewski, M., Osinska, I. & Domagala-Kulawik, J. Macrophage polarization in interstitial lung diseases. *Cent Eur J Immunol* **41**, 159-164, doi:10.5114/ceji.2016.60990 27897 [pii] (2016).

69 Prokop, S., Heppner, F. L., Goebel, H. H. & Stenzel, W. M2 polarized macrophages and giant cells contribute to myofibrosis in neuromuscular sarcoidosis. *Am J Pathol* **178**, 1279-1286, doi:S0002-9440(10)00202-6 [pii] 10.1016/j.ajpath.2010.11.065 (2011).

70 Scharl, M. *et al.* Hallmarks of epithelial to mesenchymal transition are detectable in Crohn's disease associated intestinal fibrosis. *Clin Transl Med* **4**, 1, doi:10.1186/s40169-015-0046-5 46 [pii] (2015).

71 Spector, W. G. Immunologic components of granuloma formation. Epithelioid cells, giant cells, and sarcoidosis. *Annals of the New York Academy of Sciences* **278**, 3-6 (1976).

72 Williams, W. J., James, E. M., Erasmus, D. A. & Davies, T. The fine structure of sarcoid and tuberculous granulomas. *Postgraduate medical journal* **46**, 496-500 (1970).

73 van Maarsseveen, T. C., Vos, W. & van Diest, P. J. Giant cell formation in sarcoidosis: cell fusion or proliferation with non-division? *Clin Exp Immunol* **155**, 476-486, doi:CEI3841 [pii] 10.1111/j.1365-2249.2008.03841.x (2009).

74 Hernandez-Pando, R. *et al.* Inflammatory cytokine production by immunological and foreign body multinucleated giant cells. *Immunology* **100**, 352-358 (2000).

75 Clay, H., Volkman, H. E. & Ramakrishnan, L. Tumor necrosis factor signaling mediates resistance to mycobacteria by inhibiting bacterial growth and macrophage death. *Immunity* **29**, 283-294, doi:10.1016/j.immuni.2008.06.011 (2008).

76 Bean, A. G. *et al.* Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol Mycobacterium tuberculosis infection, which is not compensated for by lymphotoxin. *Journal of immunology* **162**, 3504-3511 (1999).

77 Liu, Z. X., Noguchi, M., Hiwatashi, N. & Toyota, T. Monocyte aggregation and multinucleated giant-cell formation in vitro in Crohn's disease. The effect of cell adhesion molecules. *Scandinavian journal of gastroenterology* **31**, 706-710 (1996).

78 Okamoto, H., Mizuno, K. & Horio, T. Monocyte-derived multinucleated giant cells and sarcoidosis. *J Dermatol Sci* **31**, 119-128, doi:S0923181102001482 [pii] (2003).

79 Fehrenbach, H. *et al.* Alveolar macrophages are the main source for tumour necrosis factor-alpha in patients with sarcoidosis. *Eur Respir J* **21**, 421-428 (2003).

80 Pueringer, R. J., Schwartz, D. A., Dayton, C. S., Gilbert, S. R. & Hunninghake, G. W. The relationship between alveolar macrophage TNF, IL-1, and PGE2 release, alveolitis, and disease severity in sarcoidosis. *Chest* **103**, 832-838, doi:S0012-3692(15)41619-8 [pii] (1993).

81 Aukrust, P. *et al.* Persistent activation of the tumor necrosis factor system in a subgroup of patients with common variable immunodeficiency--possible immunologic and clinical consequences. *Blood* **87**, 674-681 (1996).

82 Mullighan, C. G., Fanning, G. C., Chapel, H. M. & Welsh, K. I. TNF and lymphotoxin-alpha polymorphisms associated with common variable immunodeficiency: role in the pathogenesis of granulomatous disease. *Journal of immunology* **159**, 6236-6241 (1997).

83 Mullighan, C. G., Marshall, S. E., Bunce, M. & Welsh, K. I. Variation in immunoregulatory genes determines the clinical phenotype of common variable immunodeficiency. *Genes Immun* **1**, 137-148, doi:10.1038/sj.gene.6363653 (1999).

84 Kamada, N. *et al.* Unique CD14 intestinal macrophages contribute to the pathogenesis of Crohn disease via IL-23/IFN-gamma axis. *J Clin Invest* **118**, 2269-2280, doi:10.1172/JCI34610 (2008).

85 Smith, A. M. *et al.* Disordered macrophage cytokine secretion underlies impaired acute inflammation and bacterial clearance in Crohn's disease. *J Exp Med* **206**, 1883-1897, doi:10.1084/jem.20091233 (2009).

86 Elliott, T. R. *et al.* Defective macrophage handling of Escherichia coli in Crohn's disease. *J Gastroenterol Hepatol* **30**, 1265-1274, doi:10.1111/jgh.12955 (2015).

87 Vazeille, E. *et al.* Monocyte-derived macrophages from Crohn's disease patients are impaired in the ability to control intracellular adherent-invasive Escherichia coli and exhibit disordered cytokine secretion profile. *J Crohns Colitis* **9**, 410-420, doi:jjv053 [pii] 10.1093/ecco-jcc/jjv053 (2015).

88 Darfeuille-Michaud, A. Adherent-invasive Escherichia coli: a putative new E. coli pathotype associated with Crohn's disease. *Int J Med Microbiol* **292**, 185-193, doi:S1438-4221(04)70097-1 [pii] 10.1078/1438-4221-00201 (2002).

89 Ryan, P. *et al.* Bacterial DNA within granulomas of patients with Crohn's disease--detection by laser capture microdissection and PCR. *Am J Gastroenterol* **99**, 1539-1543, doi:10.1111/j.1572-0241.2004.40103.x AJG40103 [pii] (2004).

90 Meconi, S. *et al.* Adherent-invasive Escherichia coli isolated from Crohn's disease patients induce granulomas in vitro. *Cell Microbiol* **9**, 1252-1261, doi:CMI868 [pii] 10.1111/j.1462-5822.2006.00868.x (2007).

91 Casanova, J. L. & Abel, L. Revisiting Crohn's disease as a primary immunodeficiency of macrophages. *J Exp Med* **206**, 1839-1843, doi:jem.20091683 [pii] 10.1084/jem.20091683 (2009).

92 Marks, D. J., Rahman, F. Z., Sewell, G. W. & Segal, A. W. Crohn's disease: an immune deficiency state. *Clin Rev Allergy Immunol* **38**, 20-31, doi:10.1007/s12016-009-8133-2 (2010).

93 Leung, S. *et al.* The cytokine milieu in the interplay of pathogenic Th1/Th17 cells and regulatory T cells in autoimmune disease. *Cellular & molecular immunology* **7**, 182-189, doi:10.1038/cmi.2010.22 (2010).

94 Chen, E. S. & Moller, D. R. Sarcoidosis--scientific progress and clinical challenges. *Nature reviews. Rheumatology* **7**, 457-467, doi:10.1038/nrrheum.2011.93 (2011).

95 Moller, D. R. *et al.* Enhanced expression of IL-12 associated with Th1 cytokine profiles in active pulmonary sarcoidosis. *Journal of immunology* **156**, 4952-4960 (1996).

96 Inui, N., Chida, K., Suda, T. & Nakamura, H. TH1/TH2 and TC1/TC2 profiles in peripheral blood and bronchoalveolar lavage fluid cells in pulmonary sarcoidosis. *The Journal of allergy and clinical immunology* **107**, 337-344, doi:10.1067/mai.2001.112273 (2001).

97 Bianco, A. & Spiteri, M. A. Peripheral anergy and local immune hyperactivation in sarcoidosis: a paradox or birds of a feather. *Clin Exp Immunol* **110**, 1-3 (1997).

98 Nemoz, G. *et al.* Impaired G-proteins and cyclic nucleotide phosphodiesterase activity in T-lymphocytes from patients with sarcoidosis. *Eur J Clin Invest* **23**, 18-27 (1993).

99 Lee, N. S. *et al.* Low levels of NF-kappaB/p65 mark anergic CD4+ T cells and correlate with disease severity in sarcoidosis. *Clin Vaccine Immunol* **18**, 223-234, doi:CVI.00469-10 [pii] 10.1128/CVI.00469-10 (2011).

100 Monteleone, G. *et al.* Interleukin 12 is expressed and actively released by Crohn's disease intestinal lamina propria mononuclear cells. *Gastroenterology* **112**, 1169-1178 (1997).

101 Garcia de Tena, J. *et al.* Active Crohn's disease patients show a distinctive expansion of circulating memory CD4+CD45RO+CD28null T cells. *J Clin Immunol* **24**, 185-196, doi:10.1023/B:JOCI.0000019784.20191.7f (2004).

102 de Souza, H. S. & Fiocchi, C. Immunopathogenesis of IBD: current state of the art. *Nature reviews. Gastroenterology & hepatology* **13**, 13-27, doi:10.1038/nrgastro.2015.186 (2016).

103 Cosmi, L., Liotta, F., Maggi, E., Romagnani, S. & Annunziato, F. Th17 and non-classic Th1 cells in chronic inflammatory disorders: two sides of the same coin. *International archives of allergy and immunology* **164**, 171-177, doi:10.1159/000363502 (2014).

104 Genovese, M. C. *et al.* Efficacy and safety of secukinumab in patients with rheumatoid arthritis: a phase II, dose-finding, double-blind, randomised, placebo controlled study. *Annals of the rheumatic diseases* **72**, 863-869, doi:10.1136/annrheumdis-2012-201601 (2013).

105 Steinman, L. A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage. *Nature medicine* **13**, 139-145, doi:10.1038/nm1551 (2007).

106 Annunziato, F. *et al.* Phenotypic and functional features of human Th17 cells. *J Exp Med* **204**, 1849-1861, doi:10.1084/jem.20070663 (2007).

107 Harbour, S. N., Maynard, C. L., Zindl, C. L., Schoeb, T. R. & Weaver, C. T. Th17 cells give rise to Th1 cells that are required for the pathogenesis of colitis. *Proceedings of the National Academy of Sciences of the United States of America* **112**, 7061-7066, doi:10.1073/pnas.1415675112 (2015).

108 Gagliani, N. *et al.* Th17 cells transdifferentiate into regulatory T cells during resolution of inflammation. *Nature* **523**, 221-225, doi:10.1038/nature14452 (2015).

109 Ten Berge, B. *et al.* Increased IL-17A expression in granulomas and in circulating memory T cells in sarcoidosis. *Rheumatology* **51**, 37-46, doi:10.1093/rheumatology/ker316 (2012).

110 Barbosa, R. R. *et al.* Primary B-cell deficiencies reveal a link between human IL-17-producing CD4 T-cell homeostasis and B-cell differentiation. *PLoS One* **6**, e22848, doi:10.1371/journal.pone.0022848 (2011).

111 Wehr, C. *et al.* The EUROclass trial: defining subgroups in common variable immunodeficiency. *Blood* **111**, 77-85, doi:10.1182/blood-2007-06-091744 (2008).

112 Sakaguchi, S., Miyara, M., Costantino, C. M. & Hafler, D. A. FOXP3+ regulatory T cells in the human immune system. *Nat Rev Immunol* **10**, 490-500, doi:10.1038/nri2785 (2010).

113 Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. & Toda, M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *Journal of immunology* **155**, 1151-1164 (1995).

114 Gambineri, E. *et al.* Clinical and molecular profile of a new series of patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome: inconsistent correlation between forkhead box protein 3 expression and disease severity. *The Journal of allergy and clinical immunology* **122**, 1105-1112 e1101, doi:10.1016/j.jaci.2008.09.027 (2008).

115 Miyara, M. *et al.* The immune paradox of sarcoidosis and regulatory T cells. *J Exp Med* **203**, 359-370, doi:10.1084/jem.20050648 (2006).

116 Oswald-Richter, K. A. *et al.* Reversal of global CD4+ subset dysfunction is associated with spontaneous clinical resolution of pulmonary sarcoidosis. *Journal of immunology* **190**, 5446-5453, doi:10.4049/jimmunol.1202891 (2013).

117 Rappl, G. *et al.* Regulatory T cells with reduced repressor capacities are extensively amplified in pulmonary sarcoid lesions and sustain granuloma formation. *Clin Immunol* **140**, 71-83, doi:10.1016/j.clim.2011.03.015 (2011).

118 Taflin, C. *et al.* FoxP3+ regulatory T cells suppress early stages of granuloma formation but have little impact on sarcoidosis lesions. *Am J Pathol* **174**, 497-508, doi:10.2353/ajpath.2009.080580 (2009).

119 Makita, S. *et al.* CD4+CD25bright T cells in human intestinal lamina propria as regulatory cells. *Journal of immunology* **173**, 3119-3130 (2004).

120 Maul, J. *et al.* Peripheral and intestinal regulatory CD4+ CD25(high) T cells in inflammatory bowel disease. *Gastroenterology* **128**, 1868-1878 (2005).

121 Mouillot, G. *et al.* B-cell and T-cell phenotypes in CVID patients correlate with the clinical phenotype of the disease. *J Clin Immunol* **30**, 746-755, doi:10.1007/s10875-010-9424-3 (2010).

122 Schubert, D. *et al.* Autosomal dominant immune dysregulation syndrome in humans with CTLA4 mutations. *Nature medicine* **20**, 1410-1416, doi:nm.3746 [pii] 10.1038/nm.3746 (2014).

123 Zeissig, S. *et al.* Early-onset Crohn's disease and autoimmunity associated with a variant in CTLA-4. *Gut* **64**, 1889-1897, doi:gutjnl-2014-308541 [pii] 10.1136/gutjnl-2014-308541 (2015).

124 Broos, C. E. *et al.* Decreased Cytotoxic T-Lymphocyte Antigen 4 Expression on Regulatory T Cells and Th17 Cells in Sarcoidosis: Double Trouble? *American journal of respiratory and critical care medicine* **192**, 763-765, doi:10.1164/rccm.201503-0635LE (2015).

125 Kakazu, T. *et al.* Type 1 T-helper cell predominance in granulomas of Crohn's disease. *Am J Gastroenterol* **94**, 2149-2155, doi:10.1111/j.1572-0241.1999.01220.x (1999).

126 Senju, M., Hulstaert, F., Lowder, J. & Jewell, D. P. Flow cytometric analysis of peripheral blood lymphocytes in ulcerative colitis and Crohn's disease. *Gut* **32**, 779-783 (1991).

127 Naser, S. A., Romero, C., Urbina, P., Naser, N. & Valentine, J. Cellular infiltration and cytokine expression correlate with fistulizing state in Crohn's disease. *Clin Vaccine Immunol* **18**, 1416-1419, doi:10.1128/CVI.05095-11 (2011).

128 Facco, M. *et al.* Sarcoidosis is a Th1/Th17 multisystem disorder. *Thorax* **66**, 144-150, doi:10.1136/thx.2010.140319 (2011).

129 Rezaei, N., Aghamohammadi, A., Kardar, G. A., Nourizadeh, M. & Pourpak, Z. T- helper 1 and 2 cytokine assay in patients with common variable immunodeficiency. *Journal of investigational allergology & clinical immunology* **18**, 449-453 (2008).

130 Parronchi, P. *et al.* Type 1 T-helper cell predominance and interleukin-12 expression in the gut of patients with Crohn's disease. *Am J Pathol* **150**, 823-832 (1997).

131 Karttunnen, R., Breese, E. J., Walker-Smith, J. A. & MacDonald, T. T. Decreased mucosal interleukin-4 (IL-4) production in gut inflammation. *Journal of clinical pathology* **47**, 1015-1018 (1994).

132 Sweiss, N. J. *et al.* Significant CD4, CD8, and CD19 lymphopenia in peripheral blood of sarcoidosis patients correlates with severe disease manifestations. *PLoS One* **5**, e9088, doi:10.1371/journal.pone.0009088 (2010).

133 Gonzalez-Juarrero, M. *et al.* Temporal and spatial arrangement of lymphocytes within lung granulomas induced by aerosol infection with Mycobacterium tuberculosis. *Infection and immunity* **69**, 1722-1728, doi:10.1128/IAI.69.3.1722-1728.2001 (2001).

134 Geboes, K. *et al.* The cellular composition of granulomas in mesenteric lymph nodes from patients with Crohn's disease. *Virchows Archiv. A, Pathological anatomy and histopathology* **409**, 679-692 (1986).

135 Fazel, S. B., Howie, S. E., Krajewski, A. S. & Lamb, D. B lymphocyte accumulations in human pulmonary sarcoidosis. *Thorax* **47**, 964-967 (1992).

136 Hermaszewski, R. A. & Webster, A. D. Primary hypogammaglobulinaemia: a survey of clinical manifestations and complications. *The Quarterly journal of medicine* **86**, 31-42 (1993).

137 Rosen, F. S., Cooper, M. D. & Wedgwood, R. J. The primary immunodeficiencies (1). *The New England journal of medicine* **311**, 235-242, doi:10.1056/NEJM198407263110406 (1984).

138 Chen, H. *et al.* Genetic regulation of pristane-induced oil granuloma responses. *International journal of experimental pathology* **91**, 472-483, doi:10.1111/j.1365-2613.2010.00732.x (2010).

139 LeBien, T. W. & Tedder, T. F. B lymphocytes: how they develop and function. *Blood* **112**, 1570-1580, doi:10.1182/blood-2008-02-078071 (2008).

140 Ueda-Hayakawa, I. *et al.* Elevated serum BAFF levels in patients with sarcoidosis: association with disease activity. *Rheumatology* **52**, 1658-1666, doi:10.1093/rheumatology/ket186 (2013).

141 Saussine, A. *et al.* Active chronic sarcoidosis is characterized by increased transitional blood B cells, increased IL-10-producing regulatory B cells and high BAFF levels. *PLoS One* **7**, e43588, doi:10.1371/journal.pone.0043588 (2012).

142 Lee, N. S. *et al.* Disturbed homeostasis and multiple signaling defects in the peripheral blood B-cell compartment of patients with severe chronic sarcoidosis. *Clin Vaccine Immunol* **18**, 1306-1316, doi:CVI.05118-11 [pii] 10.1128/CVI.05118-11 (2011).

143 Di Sabatino, A. *et al.* Depletion of immunoglobulin M memory B cells is associated with splenic hypofunction in inflammatory bowel disease. *Am J Gastroenterol* **100**, 1788-1795, doi:10.1111/j.1572-0241.2005.41939.x (2005).

144 Bogaert, D. J. *et al.* Genes associated with common variable immunodeficiency: one diagnosis to rule them all? *J Med Genet* **53**, 575-590, doi:jmedgenet-2015-103690 [pii] 10.1136/jmedgenet-2015-103690 (2016).

145 Lawrance, I. C. *et al.* Cellular and Molecular Mediators of Intestinal Fibrosis. *J Crohns Colitis*, doi:10.1016/j.crohns.2014.09.008 (2015).

146 Buttgereit, F., Saag, K. G., Cutolo, M., da Silva, J. A. & Bijlsma, J. W. The molecular basis for the effectiveness, toxicity, and resistance to glucocorticoids: focus on the treatment of rheumatoid arthritis. *Scandinavian journal of rheumatology* **34**, 14-21 (2005).

147 Joshi, P. & Dhaneshwar, S. S. An update on disease modifying antirheumatic drugs. *Inflammation & allergy drug targets* **13**, 249-261 (2014).

148 Glaesener, S. *et al.* Distinct effects of methotrexate and etanercept on the B cell compartment in patients with juvenile idiopathic arthritis. *Arthritis & rheumatology* **66**, 2590-2600, doi:10.1002/art.38736 (2014).

149 Eickenberg, S. *et al.* Mycophenolic acid counteracts B cell proliferation and plasmablast formation in patients with systemic lupus erythematosus. *Arthritis research & therapy* **14**, R110, doi:10.1186/ar3835 (2012).

150 Hanauer, S. B. *et al.* Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. *Lancet* **359**, 1541-1549, doi:10.1016/S0140-6736(02)08512-4 (2002).

151 Baughman, R. P. *et al.* Infliximab therapy in patients with chronic sarcoidosis and pulmonary involvement. *American journal of respiratory and critical care medicine* **174**, 795-802, doi:10.1164/rccm.200603-402OC (2006).

152 Keane, J. *et al.* Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. *The New England journal of medicine* **345**, 1098-1104, doi:10.1056/NEJMoa011110 (2001).

153 Franxman, T. J., Howe, L. E. & Baker, J. R., Jr. Infliximab for treatment of granulomatous disease in patients with common variable immunodeficiency. *J Clin Immunol* **34**, 820-827, doi:10.1007/s10875-014-0079-3 (2014).

154 Moreland, L. W. *et al.* Treatment of rheumatoid arthritis with a recombinant human tumor necrosis factor receptor (p75)-Fc fusion protein. *The New England journal of medicine* **337**, 141-147, doi:10.1056/NEJM199707173370301 (1997).

155 Sandborn, W. J. *et al.* Etanercept for active Crohn's disease: a randomized, double-blind, placebo-controlled trial. *Gastroenterology* **121**, 1088-1094 (2001).

156 Louie, G. H., Chitkara, P. & Ward, M. M. Relapse of sarcoidosis upon treatment with etanercept. *Annals of the rheumatic diseases* **67**, 896-898, doi:10.1136/ard.2007.078840 (2008).

157 Ehlers, S. Tumor necrosis factor and its blockade in granulomatous infections: differential modes of action of infliximab and etanercept? *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **41 Suppl 3**, S199-203, doi:10.1086/429998 (2005).

158 Ruddle, N. H. Lymphotoxin and TNF: how it all began-a tribute to the travelers. *Cytokine & growth factor reviews* **25**, 83-89, doi:10.1016/j.cytogfr.2014.02.001 (2014).

159 Di Sabatino, A. *et al.* Splenic function and IgM-memory B cells in Crohn's disease patients treated with infliximab. *Inflammatory bowel diseases* **14**, 591-596, doi:10.1002/ibd.20374 (2008).

160 Sandborn, W. J. *et al.* Abatacept for Crohn's disease and ulcerative colitis. *Gastroenterology* **143**, 62-69 e64, doi:10.1053/j.gastro.2012.04.010 (2012).

161 Kremer, J. M. *et al.* Treatment of rheumatoid arthritis by selective inhibition of T-cell activation with fusion protein CTLA4Ig. *The New England journal of medicine* **349**, 1907-1915, doi:10.1056/NEJMoa035075 (2003).

162 Hueber, W. *et al.* Secukinumab, a human anti-IL-17A monoclonal antibody, for moderate to severe Crohn's disease: unexpected results of a randomised, double-blind placebo-controlled trial. *Gut* **61**, 1693-1700, doi:10.1136/gutjnl-2011-301668 (2012).

163 Targan, S. R. *et al.* A Randomized, Double-Blind, Placebo-Controlled Study to Evaluate the Safety, Tolerability, and Efficacy of AMG 827 in Subjects With Moderate to Severe Crohn's Disease. *Gastroenterology* **143**, E26 (2012).

164 Sandborn, W. J. *et al.* Ustekinumab induction and maintenance therapy in refractory Crohn's disease. *The New England journal of medicine* **367**, 1519-1528, doi:10.1056/NEJMoa1203572 (2012).

165 Kopylov, U. *et al.* Subcutaneous ustekinumab for the treatment of anti-TNF resistant Crohn's disease--the McGill experience. *J Crohns Colitis* **8**, 1516-1522, doi:10.1016/j.crohns.2014.06.005 (2014).

166 Judson, M. A. *et al.* Safety and efficacy of ustekinumab or golimumab in patients with chronic sarcoidosis. *Eur Respir J* **44**, 1296-1307, doi:10.1183/09031936.00000914 (2014).

167 Sandborn, W. J. *et al.* Vedolizumab as induction and maintenance therapy for Crohn's disease. *The New England journal of medicine* **369**, 711-721, doi:10.1056/NEJMoa1215739 (2013).

168 Reff, M. E. *et al.* Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20. *Blood* **83**, 435-445 (1994).

169 Sweiss, N. J. *et al.* Rituximab in the treatment of refractory pulmonary sarcoidosis. *Eur Respir J* **43**, 1525-1528, doi:10.1183/09031936.00224513 (2014).

170 Papadakis, K. A., Rosenbloom, B. & Targan, S. R. Anti-CD2O chimeric monoclonal antibody (rituximab) treatment of immune-mediated thrombocytopenia associated with Crohn's disease. *Gastroenterology* **124**, 583, doi:10.1053/gast.2003.50081 (2003).


Flinders Street
9

CHAPTER 2

B-CELL DYSREGULATION IN CROHN'S DISEASE IS PARTIALLY RESTORED WITH INFLIXIMAB THERAPY

W.M.C. Timmermans [1,2], J.A.M. van Laar [1,2], T.B. van der Houwen [1,2], L.S.J. Kamphuis [1,2], S.J.W. Bartol [2], K.H. Lam [3], R.J. Ouwendijk [4], M.P. Sparrow [5], P.R. Gibson [5], P.M. van Hagen [1,2], M.C. van Zelm [2,6]

[1] Department of Internal Medicine, Erasmus MC, Rotterdam, The Netherlands
[2] Department of Immunology, Erasmus MC, Rotterdam, The Netherlands
[3] Department of Pathology, Erasmus MC, Rotterdam, The Netherlands
[4] Department of Gastroenterology, Ikazia Hospital, Rotterdam, The Netherlands
[5] Department of Gastroenterology, Alfred Hospital, Monash University and Alfred Health, Melbourne, VIC, Australia
[6] Department of Immunology and Pathology, Monash University, Melbourne, VIC, Australia

PLoS One. 2016 Jul 28;11(7):e0160103

ABSTRACT

Background and aims

B-cell depletion can improve a variety of chronic inflammatory diseases, but does not appear beneficial for patients with Crohn's disease. To elucidate the involvement of B cells in Crohn's disease, we here performed an 'in depth' analysis of intestinal and blood B-cells in this chronic inflammatory disease.

Methods

Patients with Crohn's disease were recruited to study B-cell infiltrates in intestinal biopsies (n=5), serum immunoglobulin levels and the phenotype and molecular characteristics of blood B-cell subsets (n=21). The effects of infliximab treatment were studied in 9 patients.

Results

Granulomatous tissue showed infiltrates of B lymphocytes rather than Ig-secreting plasma cells. Circulating transitional B cells and $CD21^{low}$ B cells were elevated. IgM memory B cells were reduced and natural effector cells showed decreased replication histories and somatic hypermutation (SHM) levels. In contrast, IgG and IgA memory B cells were normally present and their Ig gene transcripts carried increased SHM levels. The numbers of transitional and natural effector cells were normal in patients who responded clinically well to infliximab.

Conclusions

B cells in patients with Crohn's disease showed signs of chronic stimulation with localization to granulomatous tissue and increased molecular maturation of IgA and IgG. Therapy with TNFα-blockers restored the defect in IgM memory B-cell generation and normalized transitional B-cell levels, making these subsets candidate markers for treatment monitoring. Together, these results suggest a chronic, aberrant B-cell response in patients with Crohn's disease, which could be targeted with new therapeutics that specifically regulate B-cell function.

INTRODUCTION

The human intestinal tract contains a complex interplay between commensal bacteria, food antigens and the host immune system to limit inflammation, while preventing the translocation of intestinal microbiota. This delicate balance is disrupted in Crohn's disease, a chronic inflammatory disease characterized by transmural inflammation of the gastrointestinal tract (1). The pathogenesis of Crohn's disease is of complex nature with genetic susceptibility and dysfunction of mucosal immunity that result in a disturbed intestinal balance (2). An abnormal Th1 response is induced by dendritic cells that present commensal bacteria (3), which leads to overproduction of pro-inflammatory cytokines, including interferon-γ (IFN-γ) and tumor necrosis factor-alpha (TNF-α). In combination with impaired regulatory T cell (Treg) function, this is thought to lead to persistent inflammation in Crohn's disease (4).

In about one third of patients, histopathology of biopsy specimens show granulomas; a feature supporting the diagnosis Crohn's disease (5, 6). As early as in the 1980s, a corona of B lymphocytes around the granuloma was described (7), which parallels granulomas in patients with sarcoidosis (8). Furthermore, similar to patients with sarcoidosis (8-10), patients with Crohn's disease show signs of abnormal B-cell responses that include increased numbers of immunoglobulin (Ig)-secreting cells (11), and serum antibodies against Saccharomyces cerevisiae antibodies (ASCA) and neutrophils (ANCA) (12, 13). Being good antigen-presenters and cytokine producers, B cells can regulate T cell responses (14). Indeed, B-cells were found to affect regulatory T cell through production of IL-10 (15). However, it is not been clarified how B cells influence disease activity, because studies in murine models have reported ambiguous results, supporting either a suppressive or exacerbating role in gut inflammation (16-18).

In spite of a potential role of B cells in chronic inflammation, circulating naive B cells and class-switched memory B cells were found to be normally present in peripheral blood of patients with Crohn's disease, whereas IgM memory B cell numbers were reduced (19). IgM memory cells consist of two types; IgM-only ($CD27^+IgM^+IgD^-$) and natural effector B cells ($CD27^+IgM^+IgD^+$). While all IgM-only memory B cells originate from germinal center responses, about one-third of natural effector cells in healthy controls are derived from T-cell independent responses in the marginal zone of the spleen (20-22).

These contrasting observations did not clarify the exact role of B-cell involvement in Crohn's disease. Therefore, we here aimed to elucidate their contribution in Crohn's disease through detailed molecular analysis and immunophenotyping in locally inflamed intestinal tissue and in peripheral blood. Moreover, to evaluate candidate B-cell markers for monitoring therapeutic efficacy, we studied, the B-cell compartment after anti-TNFα therapy in patients treated with infliximab.

MATERIALS AND METHODS

PATIENTS

Clinical data and blood samples of 30 patients with Crohn's disease and 28 healthy controls were collected after written informed consent was obtained (Table 1). In addition, surplus tissue materials from diagnostic colon biopsies of 5 patients were retrospectively analyzed. This study was performed according to the Declaration of Helsinki. This study was approved by the Medical Ethics Committees of Erasmus MC (ethics approval number MEC-2011-060) and Alfred Hospital (ethics approval number 472/15) and patients were recruited from the Ikazia Hospital in Rotterdam (The Netherlands) and the Alfred Hospital in Melbourne (VIC, Australia).

IMMUNOHISTOCHEMISTRY OF GUT TISSUE BIOPSIES

Tissue slides were stained with hematoxylin and eosin. Immunohistochemistry was performed using monoclonal antibodies against CD4 (clone SP35), CD3 (2GV6), CD79a (SP18; all from Ventana, Tucson, AZ), CD8 (C8/144 B), CD20 (L26), IgG (rabbit polyclonal; all from Dako Cytomation, Glostrup, Denmark), IgA (rabbit polyclonal, Cell Marque, Rocklin, CA), CD138 (B-A38; IQ Products, Groningen, The Netherlands), and IgM (IgM88; Biogenex, Fremont, CA).

FLOWCYTOMETRY AND CELL SORTING OF BLOOD LYMPHOCYTES

Absolute counts of blood CD4 and CD8 T cells, $CD16^{+}/56^{+}$ natural killer cells, and $CD19^{+}$ B cells were obtained with a diagnostic lyse-no-wash protocol. Eight-color flow cytometric analysis was performed as described previously to detect transitional, naive mature, six memory B cell subsets, plasmablasts and $CD21^{low}CD38^{dim}$ cells (Supplemental Figure 1) on a 3-laser FACS LSRII with standardized configuration according to Euroflow protocols (BD Biosciences, San Jose, CA) (23). Detailed analysis of B cell subsets was performed with IgM-HorV450 (G20-127; BD), IgD-biotin (IA6-2), IgG-PE (G18-145), CD19-PE-Cy7 (SJ25C1), CD19-PerCP-Cy5.5 (SJ25C1), CD21-PE-Cy7 (B-ly4), CD27-PerCP-Cy5.5 (L128), CD27-APC (L128), CD38-APC-H7 (HB7; all from BD Biosciences, San Jose, CA, USA) and IgA-FITC (IS11-8E10; Miltenyi-Biotec GmbH, Germany) (24). Biotinylated antibodies were visualized with streptavidin-Pac.Orange (Invitrogen).

Naive mature and natural effector B cells were high-speed cell sorted to greater than 95% purity on a FACSAria I (BD Biosciences), as described previously (25).

TABLE 1. Clinical and basic immunological characteristics of patients with Crohn's disease.

Patient	Gender	Age (yr)	Disease duration (yr)	Medication	Surgery	Granuloma	B-cells	T-cells	NK-cells	IgG	IgG1	IgG2	IgG3	IgG4	IgA	IgA1	IgA2	IgM
1	F	30	0	None	No	Yes	**91**	1,200	270	ND	ND	ND	ND	ND	ND	ND	ND	ND
2	F	25	3	None	Yes	No	197	1,100	310	ND	ND	ND	ND	ND	ND	ND	ND	ND
3	M	23	4	None	No	No	208	1,575	220	ND	ND	ND	ND	ND	ND	ND	ND	ND
4	M	34	13	5-ASA	Yes	No	**430**	1,120	**430**	**6.8**	**4.8**	1.9	0.2	1.0	2.8	2.0	**1.4**	1.4
5	M	43	10	5-ASA	Yes	Yes	**470**	1,620	270	10.1	7.1	3.8	0.9	**<0.06**	1.7	1.2	0.2	0.7
6	M	63	5	5-ASA	Yes	No	160	**2,190**	310	10.0	6.2	4.8	0.8	0.4	**4.9**	**3.6**	**1.6**	0.7
7	M	35	22	None	Yes	No	**90**	**470**	220	9.0	5.2	5.3	0.6	0.1	**4.3**	**2.9**	**2.4**	0.7
8	M	33	2	5-ASA	No	Yes	220	1,260	190	11.1	8.3	2.7	0.9	0.5	3.6	**2.6**	**0.8**	0.5
9	F	22	7	None	No	No	250	1,110	270	14.9	11.1	4.0	0.8	0.4	2.6	1.8	0.3	2.3
10	F	27	0	5-ASA	No	Yes	**590**	1,790	230	9.2	5.7	3.7	0.5	1.2	1.8	1.3	0.3	1.1
11	F	62	21	5-ASA	Yes	Yes	160	1,090	220	7.8	**4.8**	3.7	0.5	**<0.06**	3.4	2.3	**1.0**	0.6
12	F	26	8	5-ASA	No	No	260	750	150	10.6	8.2	3.1	0.2	0.2	1.5	1.1	0.4	0.6
13	F	61	11	5-ASA	Yes	No	210	1,600	160	15.1	**13.0**	2.5	0.7	0.1	2.1	1.5	0.3	1.6
14	F	48	34	5-ASA	Yes	Yes	180	1,220	100	8.4	7.3	**0.9**	0.5	**<0.06**	2.7	1.8	**1.0**	0.7
15	M	45	6	5-ASA	No	No	380	**2,060**	**820**	**17.3**	10.7	**8.3**	0.4	**2.2**	**5.8**	**4.3**	**1.3**	0.7
16	F	35	1	None	No	No	333	**1,990**	250	10.7	7.1	5.3	0.6	**<0.06**	0.7	**0.5**	0.2	1.2
17	F	31	11	None	No	No	230	**3,250**	250	9.5	8.5	**1.4**	0.7	**<0.06**	1.3	1.0	0.4	1.3
18	F	35	8	None	No	Yes	350	1,120	180	**16.4**	**12.5**	6.0	1.0	0.2	3.3	2.4	0.5	1.4
19	F	41	23	5-ASA	Yes	No	110	1,330	150	8.8	5.8	4.1	0.4	**<0.06**	2.5	1.6	**1.1**	1.2
20	F	53	0	None	Yes	No	**870**	**2,500**	260	**6.3**	**4.3**	2.9	0.5	0.1	0.8	0.6	0.3	ND
21	M	39	3	5-ASA	No	No	**680**	1,580	**660**	15.8	**13**	3.5	0.8	1.1	1.6	1.1	0.4	1.0

TABLE 1. Continued

Patient	Gender	Age (yr)	Disease duration (yr)	Medication	Surgery	Granuloma	B-cells	T-cells	NK-cells	IgG	IgG1	IgG2	IgG3	IgG4	IgA	IgA1	IgA2	IgM
22	F	34	14	IFX, 5-ASA	No	No	240	1,730	160	11.7	9.0	2.5	0.4	0.1	1.4	1.1	0.1	1.3
23	F	51	14	IFX, AZA	Yes	Yes	150	970	110	8.7	6.8	2.1	0.4	0.1	2.3	1.8	0.2	1.1
24	M	31	4	IFX	No	No	200	1,130	**80**	9.0	5.0	4.0	0.7	0.7	2.3	1.7	0.3	0.5
25	M	58	37	IFX, 5-ASA, AZA	Yes	No	200	**2,030**	**30**	**16.9**	**13.3**	2.8	0.6	0.1	2.6	1.9	0.2	0.9
26	F	48	19	IFX	Yes	No	370	**2,400**	**780**	9.6	5.1	4.4	0.4	**<0.06**	3.1	2.1	**1.4**	1.4
27	F	22	4	IFX	Yes	No	**790**	1,760	220	ND	ND	ND	ND	ND	ND	ND	ND	ND
28	F	33	15	IFX, AZA	Yes	Yes	128	**513**	**21**	ND	ND	ND	ND	ND	ND	ND	ND	ND
29	F	46	9	IFX	Yes	No	120	**460**	**28**	ND	ND	ND	ND	ND	ND	ND	ND	ND
30	M	22	2	IFX, AZA	No	No	199	855	**22**	ND	ND	ND	ND	ND	ND	ND	ND	ND

Lymphocyte subsets are provided in cells/µL, Immunoglobulin levels in g/L. Abnormal values are depicted in bold font. Definition of abbreviations: F, female; M, male 5-ASA, 5-aminosalicylic acid; IFX, infliximab; AZA, azathioprine. Normal values: B cells: 100-400 cells/µl; T cells: 700-1900 cells/µl; NK cells; 100-400 cells/µl; IgG: 7-16 g/L; IgG1: 4.9-11.4 g/L; IgG2: 1.50-6.4 g/L; IgG3: 0.20-1.10 g/L; IgG4: 0.080-1.40 g/L; IgA: 0.70-4.0 g/L; IgA1: 0.6-2.4 g/L; IgA2: 0.1-0.6 g/L; IgM 0.4-2.3 g/L.

QUANTIFICATION OF SERUM IMMUNOGLOBULIN LEVELS

Serum IgM, IgG, and IgA levels were measured with an immunoturbidimetric method (Hitachi Analyzer; Roche, Basel, Switzerland). IgG and IgA subclasses were determined using the immunonephelometric method (Sanquin, Amsterdam, The Netherlands).

MOLECULAR ANALYSIS OF REPLICATION HISTORY AND IMMUNOGLOBULIN HEAVY CHAIN (IGH) TRANSCRIPTS

IGHA and *IGHG* transcripts were amplified from PBMC cDNA of patients with Crohn's disease (n=4) and healthy controls (n=4). *IGHV3* and *IGHV4* leader primers and consensus Cα or Cγ reverse primers were used (22).

DNA was isolated from sorted naive mature and natural effector B cells of patients with Crohn's disease (n=4) to analyze the replication history with the kappa-deleting recombination excision circle assay as described previously (25). In addition, *IGH* gene rearrangements were amplified from DNA of sorted natural effector B cells. PCR products were cloned into the pGEM-T easy vector (Promega, Madison, WI) and prepared for sequencing on an ABIPRISM 3130XL (Applied Biosystems, Carlsbad, CA). Obtained sequences were analyzed with IMGT database (http://imgt.cines.fr), Joinsolver (https://joinsolver.niaid.nih.gov) and Bayesian estimation of Antigen-driven SELectIoN (BASELINe; http://selection.med.yale.edu/baseline/). IgA and IgG receptor subclasses were determined using the *IGH* reference sequence (NG_001019).

STATISTICS

Statistical analyses were performed using the Mann-Whitney test (SPSS version 18.0), $\chi 2$ test or Spearman correlation as indicated in Figure legends. A P-value <0.05 was considered statistically significant.

RESULTS

CLINICAL AND BASIC IMMUNOLOGICAL CHARACTERIZATION OF PATIENTS

In this study, 30 patients with biopsy-confirmed Crohn's disease (11 males) were included with a mean age of 38.5 yr (range 22-62 yr; Table 1). Patients 1-21 had not received immunosuppressive drugs for at least three months prior to inclusion. Of these 21 patients, 12 patients received 5-ASA medication, 9 were without any medication for Crohn's disease and 11/21 patients had received systemic immunosuppressive medication in the past. All patients had clinically mild to moderate disease without a

need for systemic immune suppressive treatment at the time of study inclusion. Patients 22-30 received infliximab treatment for >6 weeks at study inclusion and were clinically good responders. In 9/30 patients, granulomas were previously detected in ileal or colon biopsies and 16/30 patients had a history of surgical resection of the gut. The average duration of disease at study inclusion was 10.3 years (range 0-34 year). Mean values of B, T and NK cells, as well as mean serum IgM, IgG and IgA levels were within the normal range. The average IgA2 serum level of the patients was increased as compared to controls (0.7 g/L; range 0.1-2.4; normal range 0.1-0.6), with 9/23 patients having levels above the normal range.

B CELL LOCALIZATION AROUND GRANULOMAS

All colon tissue biopsies showed inflammation compatible with Crohn's disease. Haematoxylin and eosin-staining of the granulomas did not show any sign of necrosis. T cells were easily detectable with stainings for CD3, CD4 or CD8, and were located throughout the inflamed tissue, both inside and outside the granulomas (Figure 1). In agreement with previous observations (7), $CD4^+$ T cells were more numerous than $CD8^+$ T cells with a ratio of 4:1. $CD20^+$ B cells were detectable in intestinal biopsies, but these were restricted to normal lymphoid follicles and were very sparse in the non-granulomatous inflamed tissue (Figure 1). However, directly surrounding the granulomas, B cells were numerous as visualized with CD20 or CD79a stainings (Figure 1).

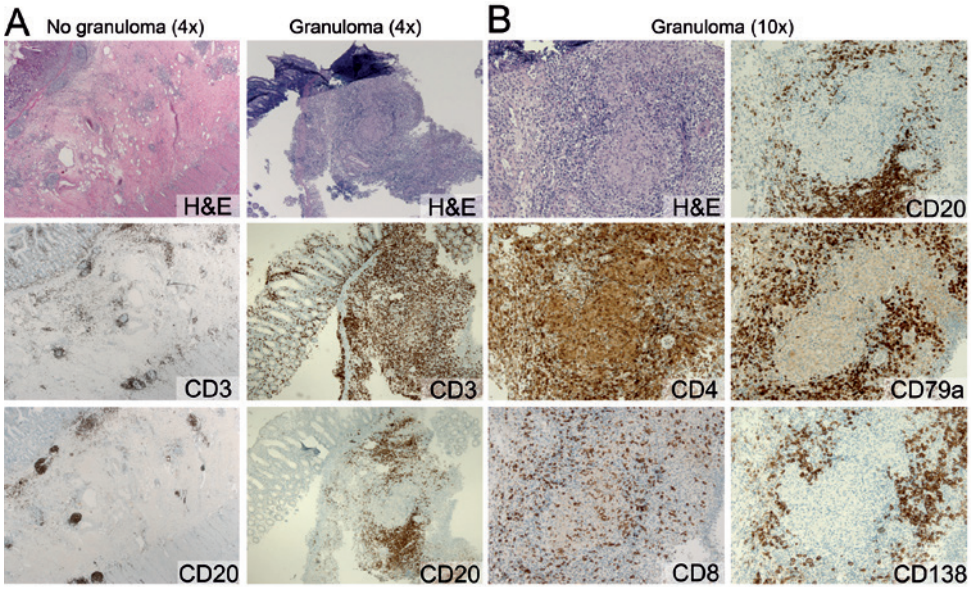


FIGURE 1. B-cells accumulate around granulomas in affected colon tissue in Crohn's disease. **A,** Representative images of colon biopsies with granulomas and non-granulomatous tissue in two patients with Crohn's disease. **B,** Magnifications of granulomatous tissue from panel A.

Plasma cells are abundantly present in the human gut tissue of healthy individuals, with IgA as their major product (26, 27). Tissue sections from our patients with Crohn's disease showed numerous CD138$^+$ plasma cells, both in inflamed and in non-inflamed regions. These plasma cells were not specifically localized near granulomas, but were distributed over the gut tissue with the majority secreting IgA and smaller fractions IgG or IgM (Supplemental Figure 2). The specific localization of B cells surrounding granulomas indicates involvement of B cells in the immunopathogenesis of granulomatous inflammation in Crohn's disease.

ABNORMALITIES IN BLOOD B CELL SUBSETS IN PATIENTS WITH CROHN'S DISEASE

To study whether local intestinal inflammation affected B cells systemically, we studied blood B-cell subsets in 21 patients with Crohn's disease. Flowcytometric analysis revealed normal numbers of total CD19$^+$ B-cells in patients (n=21) as compared with healthy controls (n=28). Further subsetting of these CD19$^+$ B cells (Figure 2A) revealed significantly increased numbers of CD38^{high}CD24^{high} transitional B cells (P=0.009), while CD27$^-$IgD$^+$ naive mature B cells were normally present (Figure 2B). Within the antigen-experienced B-cell compartment, IgM$^+$ memory B cells were low with CD27$^+$IgM$^+$IgD$^+$ natural effector B cells being significantly decreased (P<0.001), IgM-only B cells (CD27$^+$IgM$^+$IgD$^-$) not-significantly decreased (P = 0.06). The numbers of class-switched

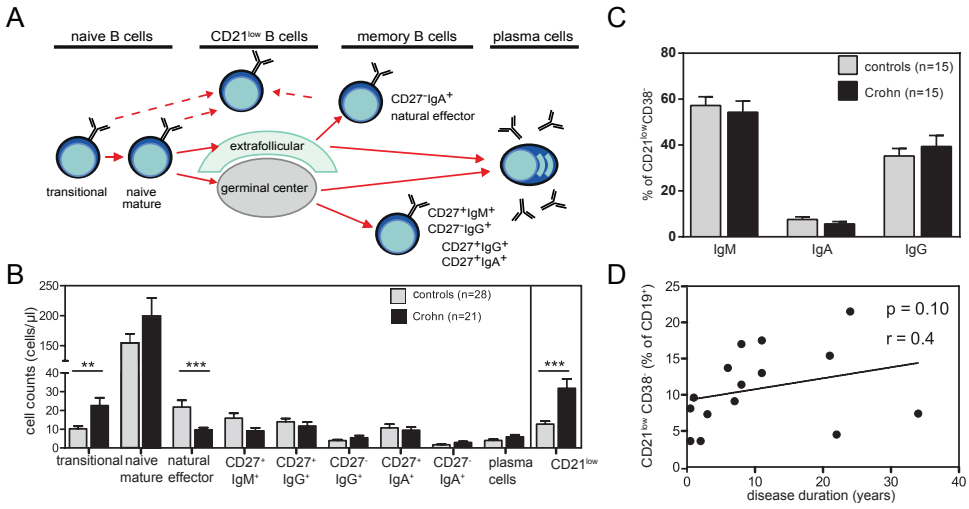


FIGURE 2. Composition of the blood B-cell compartment in patients with Crohn's disease.

A. Schematic overview of peripheral B-cell subsets. **B.** Average numbers of blood B cell subsets of 21 patients affected with Crohn's disease (black bars) and 28 healthy controls (light grey bars). **C.** Distribution of IgM, IgA and IgG within CD21^{low} in patients and controls **D.** Total CD21^{low} B cells in relation to disease duration. Statistical analyses were performed with the Mann-Whitney test or Spearman correlation; *, P<0.05; **, P<0.01.

B cell subsets ($CD27^+IgG^+$, $CD27^-IgG^+$, $CD27^+IgA^+$ and $CD27^-IgA^+$) and plasma blasts were similar between patients and controls. Large fractions of the patients' B cells showed low CD21 expression levels and these numbers were significantly higher than in healthy controls (P<0.0001). The increase was not related to disease duration (P=0.10; Figure 2D), and a large fraction of these $CD21^{low}$ B cells were Ig class switched to IgA or IgG, suggestive of an origin from antigen-experienced B cells (Figure 2C).

To study whether the abnormalities in transitional, natural effector and $CD21^{low}$ B cells were associated with surgical treatment and current or past medication, additional analyses were performed following division of the total 21 patients into patients with (n=10) or without surgical resection (n=11), into patients currently treated with (n=12) or without 5-ASA medication (n=9) and patients with (n=11) and without (n=10) a history of systemic medication. All three analyses revealed similar patterns for the separate patient groups (Supplemental Figure 3), thereby excluding differential effects of these treatments on the blood B-cell compartment.

IMPAIRED GENERATION OF NATURAL EFFECTOR B CELLS

Our flowcytometric analysis showed decreased numbers of natural effector B cells in peripheral blood of patients with Crohn's disease, line with previous findings (19). To study whether the decline was due to impaired generation of these cells, we analyzed the replication history and somatic hypermutation (SHM) levels in purified cells from four patients (Patient 15, 16, 18 and 19). Naive mature B cells of patients and controls showed a similar replication history of 1-2 cell divisions (25). However, the patients' natural effector B cells showed a replication history of only 2 cell division versus 9 in controls (P=0.002; Figure 3A). These natural effector B cells carried diverse *IGH* gene rearrangements, with shorter *IGH*-CDR3 sizes than in naive B cells, which is a typical feature of antigen-experienced B cells (Figure 3D) (22). Still, the majority of rearrangements amplified from the patients carried unmutated *IGHV* genes (28 of 51 unique rearranged *IGHV*). Moreover, the overall SHM levels were significantly lower than in controls (P<0.0001; Figure 3B), and hardly higher than in naive mature B cells. The few mutations in patients' Ig genes were normally targeted (Supplemental Table 1). However, on top of their low numbers, the mutations in complementarity determining regions (CDR) were not selected for amino acid replacements as is typical seen in healthy controls (P<0.0001; Figure 3C). Thus, IgM^+IgD^+-expressing memory B cells in patients with Crohn's disease are not only decreased in number, they also display a defects in replication history and SHM.

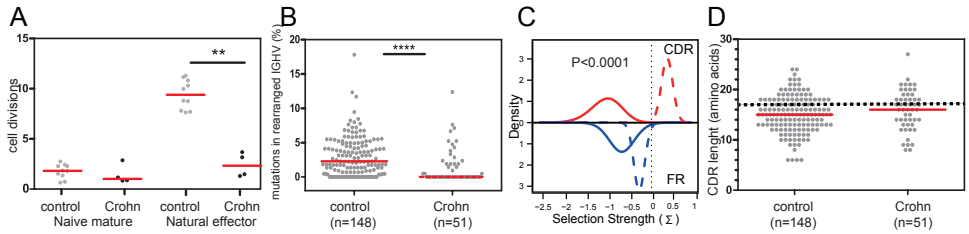


FIGURE 3. Replication history and SHM levels in *IGHV* genes of natural effector B cells.

A. Replication history of naïve and natural effector B cells as assessed using the KREC assay (25). **B.** *IGHV* mutation frequencies in rearranged *IGH* genes of natural effector B cells in patients and controls (total numbers of sequences indicated between brackets). Grey dots represent unique sequences; red lines represent median values. **C**. Selection for replacement mutation in IGHV-CDR (red line) and IGHV-FR regions (blue lines) as determined with the BASELINe program (28, 29). Solid lines represent patients; dashed lines represent healthy controls. Selection Strengths >0 indicate positive selection. **D.** IGH-CDR3 size distributions. All individual sizes are indicated as grey dots, red lines representing median values. The dashed line represents median values for centroblasts and centrocytes. Sorted cells were analyzed from patients 15, 16, 18 and 19. Controls were published previously (30, 31). Statistical analysis was performed with the Mann-Whitney test; *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001.

INCREASED SHM LEVELS IN IG GENES OF SWITCHED MEMORY B CELLS

In contrast to IgM$^+$ memory B cells, Ig class-switched memory B cells were normally present in blood of patients with Crohn's disease (Figure 2) (19). Their IgA and IgG transcripts displayed a diverse usage of *IGHV3* and *IGHV4* subgroups with CDR3 size distributions similar to those of controls and typical for antigen-experienced B cells with a median of 15 amino acids (Supplemental Figure 5). These transcripts showed high levels of SHM, which appeared to be normally targeted to the typical sequence motifs (Supplemental Table 1). In addition, nucleotide substitution spectra and transition/transversion ratios did not differ between patients and controls. To determine whether these transcripts showed signs of antigen selection, we analyzed selection for replacement mutations using the BASELINE program. Similar to healthy controls, sequences derived from patients with Crohn's disease showed positive selection for replacement mutations in CDR and negative selection in framework regions (FR) (Supplemental Figure 5C).

To study whether the high SHM levels were the result of altered IgG and IgA subclass usage, we analyzed these in the rearranged transcripts (32, 33). Patients with Crohn's disease showed increased IgA1 and IgG2 usage, to the expense of IgA2 and IgG3 (Figure 4B). Still, these altered distributions did not underlie the difference in SHM levels. IgA1 and IgA2, as well as IgG1 and IgG2 transcripts of the patients carried more SHM than those of controls (Figure 4C). More specifically, a substantial fraction of IgA2 transcripts from controls was hardly mutated, and this fraction was nearly absent in patients with Crohn's disease. In conclusion, patients with Crohn's disease show increased levels of SHM with otherwise normal targeting and selection for replacement mutations. This was independent of the concomitant reduction in IgA2 and IgG3 subclass usage.

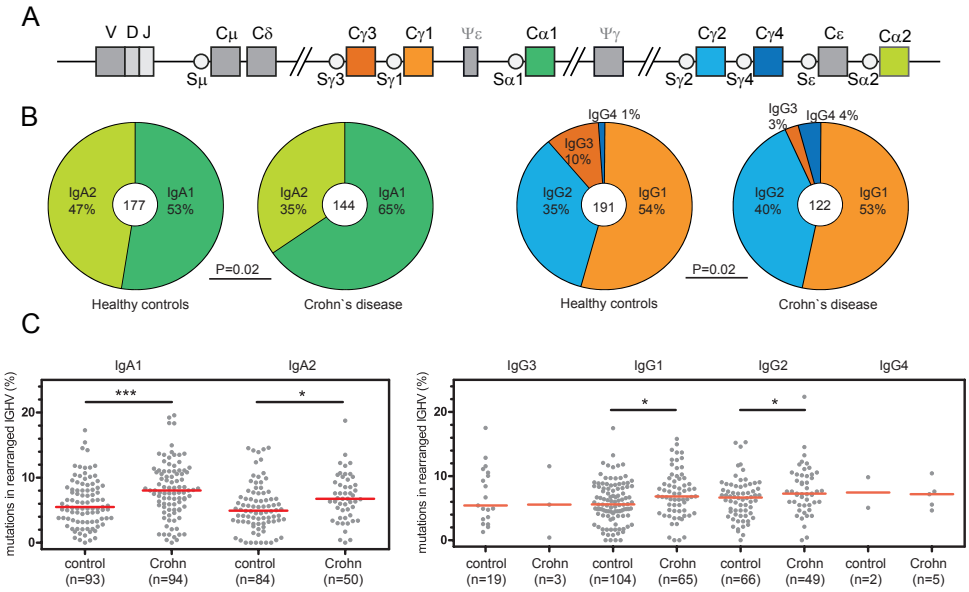


FIGURE 4. IgA and IgG subclass analysis.

A. Schematic representation of the constant region of the human IGH locus. **B.** Distribution of IgA and IgG subclass use in switched transcripts of healthy controls and patients with Crohn's disease. Total numbers of analyzed sequences are indicated in the middle of the plots. χ^2 Test was performed to analyze differences in distributions. **C.** Combined *IGHV* mutation frequencies in IgA and IgG transcripts in patients and controls (total numbers of sequences indicated between brackets). Grey dots represent unique sequences; red lines represent median values. Statistical analysis was performed with the Mann-Whitney test; *, P<0.05; **, P<0.01; ***, P<0.001.

NORMALIZATION OF BLOOD B-CELL SUBSETS IN INFLIXIMAB-TREATED PATIENTS

Previous observations indicated normalization of spleen function and levels of circulating IgM+ memory B cells in Crohn's disease patients following infliximab therapy (34). To study whether successful treatment normalized the total peripheral B-cell compartment, we phenotyped blood B cells in 9 patients that were receiving infliximab (patients 21-30; Table 1). Infliximab was administered once every eight weeks for a long period of time (range 8 months-10 years), and all patients were in clinical response after treatment. In contrast to patients not receiving infliximab, transitional B cells and natural effector B cells were normalized to levels comparable with healthy controls (Figure 5A). However, IgM-only B cell numbers were still low, and $CD21^{low}$ B cells remained increased as compared to healthy controls. Within $CD21^{low}$, the IgM, IgA and the IgG expressing subsets were higher in number than in healthy controls. Patients treated with infliximab showed a further increase in the IgA subset, with an accompanied (non-significant) decrease in IgM (Figure 5B). A substantial fraction of the $CD21^{low}$ subset expressed CD27, and $CD27^+$ and $CD27^-$ was similar between controls and patients (Supplemental

Figure 4A). In absolute numbers, both fractions were elevated in patients. Thus, patients with Crohn's disease show systemic abnormalities in their B cell compartments, which appear almost completely recovered by successful infliximab treatment.

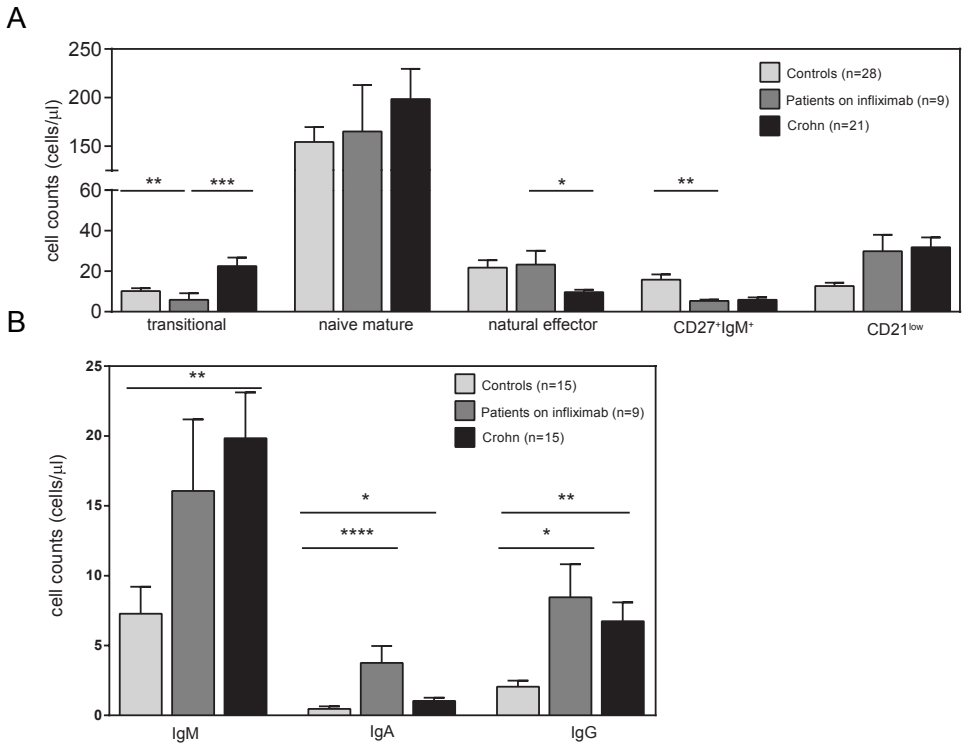


FIGURE 5. Effects of infliximab on blood B-cell and CD21^{low} compartment.

A. Blood B-cell compartments in patients under treatment with infliximab. **B.** Absolute total numbers of IgM, IgA and IgG with low CD21 expression in controls, patients and patients under treatment with infliximab. Bars represent mean values ±SEM. Statistical analysis was performed with the Mann-Whitney test; *, P<0.05; **, P<0.01; ***, P<0.001.

DISCUSSION

In this study, we demonstrate that patients with Crohn's disease have an infiltration of B cells around granulomas and an altered B-cell compartment in the peripheral blood. While IgM memory B-cell formation was impaired, Ig class switched B cells showed molecular sings of chronic stimulation. Importantly, alterations in the peripheral B-cell compartment normalized after treating inflammation effectively with TNFα-blockers.

Our findings of B cells surrounding granulomas in Crohn's disease extend previous observations from the 1980s (7), and more recent ones from pediatric patients with Crohn's disease with *NOD2* gene mutations (35). These B cells appear to be lymphocytes

and not plasma cells and localize specifically around the granulomas. Still, the origin and types of B cell subsets remain unclear. Large amounts of B cells were also found to be present around the granulomas in sarcoidosis (8), and consequently researchers have evaluated treatment with anti-CD20 therapy (e.g. with rituximab) (36). It is possible that these B cells are crucial for the formation of granulomas. This is supported by studies in mouse models that were capable of granuloma formation in the absence of T cells, but not in the absence of B cells (37). Furthermore, granulomas are found in a large fraction of patients with antibody deficiencies in the presence of B cells (esp. Common Variable Immunodeficiency; CVID), while these have not been reported in patients with X-linked agammaglobulinemia, who lack circulating B cells due to a block in differentiation of precursor B cells (38). How these B cells would function in the formation of granulomas remains unclear. B-cell depletion therapy seems to induce and exacerbate colitis (39, 40), while immunoglobulin substitution can induce rapid dampening of inflammation in patients with Crohn's disease (41). Thus, it is well-possible that the local B cells have a regulatory function to control inflammation (42).

Our patients showed alterations in blood B cell subsets in absence of systemic immunosuppressive therapy. One of these was a strong reduction in circulating IgM+ memory B cells, which was the result of impaired generation rather than increased loss, because the few remaining IgM memory 'natural effector' B cells showed severely reduced replication history, SHM levels and absence of selection for replacement mutations in CDR. The loss of IgM memory B cells was previously attributed to impaired spleen function (19). However, a large fraction of these 'natural effector' B cells is dependent on T-cell help and more likely originates from germinal center reactions (21, 22). Considering the strongly decreased natural effector B-cell numbers in our patients, it is therefore likely that in addition to IgM responses in the spleen, also germinal center responses are impaired in the generation of IgM^+ memory B cells in patients with Crohn's disease.

In contrast to IgM^+ memory, transitional and $CD21^{low}$ B-cell numbers were increased in our patients. Higher numbers of transitional cells were previously observed in patients with other chronic inflammatory diseases, including sarcoidosis and SLE (8, 9, 43). This increase could reflect increased B-cell output from the bone marrow. Still, this did not result in higher numbers of circulating mature B cells and might be due to inability of these transitional B cells to further mature. $CD21^{low}$ B cells are peculiar cells that have been described to be functionally anergic with the downregulation of CD21 suppressing their responsiveness and decreasing their survival (22, 44). The increase in transitional B cells could therefore be a compensation for the loss of mature B cells through downregulation of CD21. While $CD21^{low}$ B cells are scarce in healthy controls, their numbers are increased during infections, autoimmune diseases (22, 45, 46), CVID with autoimmunity and Down syndrome (44, 46-48). As these cells were not increased in patients with sarcoidosis (8), $CD21^{low}$ B cells could represent a marker of distinct pathophysiology between these two granulomatous inflammatory diseases.

The numbers of natural effector B cells normalized under infliximab therapy, an observation that was made previously as well and was associated with restoration of spleen function (34). More recently, Li and colleagues also confirmed these low numbers of pre-switched memory B cells in inflammatory bowel disease and its restoration with TNFα-blockers (49). Thus, infliximab therapy either directly or indirectly by dampening inflammation restores IgM memory in patients with Crohn's disease. Whether natural effector B cells can predict successful therapeutic outcome would need to be investigated in future studies with longitudinal follow-up of patients. Treatment with 5-ASA did not show this effect on the B-cell compartment. This could be due to the difference in therapeutic mechanisms or the merely local application of 5-ASA medication in contrast to systemic effects of infliximab. Alternatively, infliximab can induce and maintain mucosal healing (50). Furthermore, the $CD21^{low}$ population was the aberrant B-cell subset in our patient group that did not normalize during treatment with infliximab, suggesting that the process to downregulate CD21 is either not affected by TNFα-blockers, or is maintained to dampen inflammation.

A large fraction of $CD21^{low}$ B cells was Ig class switched, suggesting their origin from memory B cells. Indeed, the increased SHM levels in IgA and IgG transcripts reflected abnormally high or strong activation of these class-switched memory cells. SHM levels are tightly regulated and even in individuals continuously exposed to parasites these are not increased (32, 33). Notably, IgA transcripts in patients with Crohn's disease were highly mutated, and the frequencies of hypomutated transcripts were lower than in healthy controls. A substantial fraction of blood IgA+ memory B cells carries polyreactive immunoglobulins. These are typically highly mutated and bind strongly to mucosa-colonizing bacteria (51). Despite the high SHM levels, the Ig transcripts from patients with Crohn's disease did not show signs of additional selection for replacement mutations in CDR regions. This is suggestive of a lack in additional affinity maturation, and the result of abnormal and chronic activation in patients with Crohn's disease and in previously studied sarcoidosis patients (8). Despite the signs of chronic stimulation, total numbers of IgA and IgG memory B cells were not increased in blood of patients with Crohn's disease. This is potentially due to their infiltration into tissue. Alternatively, these cells could be silenced by downregulating CD21 expression. This would make the cells more susceptible to cell death and would explain, at least in part, the expansion of the $CD21^{low}$ B cell population.

Our study demonstrates distinct B-cell maturation alterations in both local inflamed tissue and in peripheral blood of patients with Crohn's disease. These effects were independent of 5-ASA treatment or past systemic therapy and surgical resections, and seemed homogeneous in our study population. Especially the Ig class-switched B cells show signs of chronic stimulation, while the generation of IgM memory B cells is impaired. Moreover, clinical improvement is heralded by normalization of the elevated circulating transitional and natural effector B cells in response to TNFα-blockers. Thus,

through dissection of the local and systemic B cell compartments, this study provides new insights into their role in chronic inflammation. Specifically, blood B-cell deviations could represent good markers to predict treatment success before or early after start of infliximab or other novel therapeutics in Crohn's disease.

ACKNOWLEDGEMENTS

The authors thank Dr. D. van den Heuvel (Erasmus MC, Rotterdam, The Netherlands) for her advice on analysis, and the (research)nurses and gastroenterologists of the Department of Gastroenterology at the Ikazia Hospital (Rotterdam, The Netherlands) and the Department of Gasteroenterology at the Alfred Hospital for support with patient inclusion. This study was performed in the framework of the Molecular Medicine Postgraduate School of the Erasmus MC.

REFERENCES

1. Baumgart DC, Sandborn WJ. Crohn's disease. Lancet. 2012;380(9853):1590-605.
2. Baumgart DC, Carding SR. Inflammatory bowel disease: cause and immunobiology. Lancet. 2007;369(9573):1627-40.
3. Geremia A, Biancheri P, Allan P, Corazza GR, Di Sabatino A. Innate and adaptive immunity in inflammatory bowel disease. Autoimmunity reviews. 2014;13(1):3-10.
4. Hisamatsu T, Kanai T, Mikami Y, Yoneno K, Matsuoka K, Hibi T. Immune aspects of the pathogenesis of inflammatory bowel disease. Pharmacol Ther. 2013;137(3):283-97.
5. Heresbach D, Alexandre JL, Branger B, Bretagne JF, Cruchant E, Dabadie A, et al. Frequency and significance of granulomas in a cohort of incident cases of Crohn's disease. Gut. 2005;54(2):215-22.
6. Lennard-Jones JE, Lockhart-Mummery HE, Morson BC. Clinical and pathological differentiation of Crohn's disease and proctocolitis. Gastroenterology. 1968;54(6):1162-70.
7. Geboes K, van den Oord J, De Wolf-Peeters C, Desmet V, Rutgeerts P, Janssens J, et al. The cellular composition of granulomas in mesenteric lymph nodes from patients with Crohn's disease. Virchows Archiv A, Pathological anatomy and histopathology. 1986;409(5):679-92.
8. Kamphuis LS, van Zelm MC, Lam KH, Rimmelzwaan GF, Baarsma GS, Dik WA, et al. Perigranuloma localization and abnormal maturation of B cells: emerging key players in sarcoidosis? Am J Respir Crit Care Med. 2013;187(4):406-16.
9. Saussine A, Tazi A, Feuillet S, Rybojad M, Juillard C, Bergeron A, et al. Active chronic sarcoidosis is characterized by increased transitional blood B cells, increased IL-10-producing regulatory B cells and high BAFF levels. PLoS One. 2012;7(8):e43588.
10. Lee NS, Barber L, Akula SM, Sigounas G, Kataria YP, Arce S. Disturbed homeostasis and multiple signaling defects in the peripheral blood B-cell compartment of patients with severe chronic sarcoidosis. Clin Vaccine Immunol. 2011;18(8):1306-16.
11. Sieber G, Herrmann F, Zeitz M, Teichmann H, Ruhl H. Abnormalities of B-cell activation and immunoregulation in patients with Crohn's disease. Gut. 1984;25(11):1255-61.
12. Quinton JF, Sendid B, Reumaux D, Duthilleul P, Cortot A, Grandbastien B, et al. Anti-Saccharomyces cerevisiae mannan antibodies combined with antineutrophil cytoplasmic autoantibodies in inflammatory bowel disease: prevalence and diagnostic role. Gut. 1998;42(6):788-91.
13. van Schaik FD, Oldenburg B, Hart AR, Siersema PD, Lindgren S, Grip O, et al. Serological markers predict inflammatory bowel disease years before the diagnosis. Gut. 2013;62(5):683-8.
14. Harris DP, Haynes L, Sayles PC, Duso DK, Eaton SM, Lepak NM, et al. Reciprocal regulation of polarized cytokine production by effector B and T cells. Nat Immunol. 2000;1(6):475-82.
15. Mizoguchi A, Bhan AK. A case for regulatory B cells. J Immunol. 2006;176(2):705-10.
16. Mizoguchi E, Mizoguchi A, Preffer FI, Bhan AK. Regulatory role of mature B cells in a murine model of inflammatory bowel disease. International immunology. 2000;12(5):597-605.
17. Olson TS, Bamias G, Naganuma M, Rivera-Nieves J, Burcin TL, Ross W, et al. Expanded B cell population blocks regulatory T cells and exacerbates ileitis in a murine model of Crohn disease. J Clin Invest. 2004;114(3):389-98.
18. LeBien TW, Tedder TF. B lymphocytes: how they develop and function. Blood. 2008;112(5):1570-80.

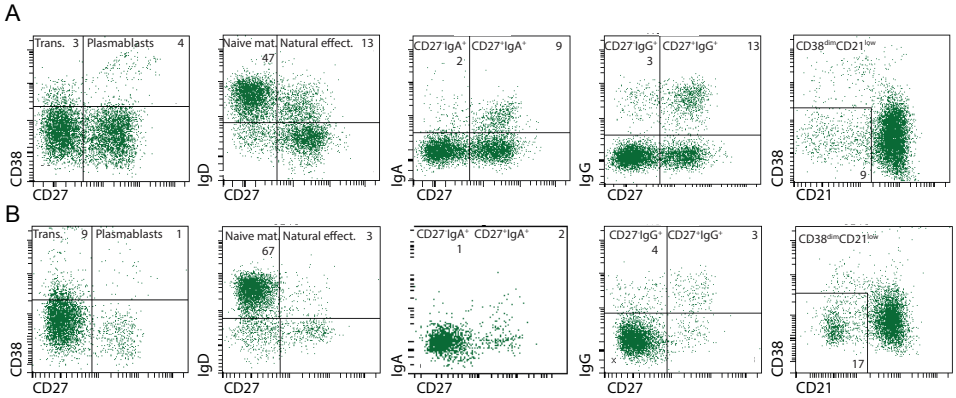
19. Di Sabatino A, Rosado MM, Ciccocioppo R, Cazzola P, Morera R, Corazza GR, et al. Depletion of immunoglobulin M memory B cells is associated with splenic hypofunction in inflammatory bowel disease. The American journal of gastroenterology. 2005;100(8):1788-95.
20. Kruetzmann S, Rosado MM, Weber H, Germing U, Tournilhac O, Peter HH, et al. Human immunoglobulin M memory B cells controlling Streptococcus pneumoniae infections are generated in the spleen. J Exp Med. 2003;197(7):939-45.
21. Weller S, Braun MC, Tan BK, Rosenwald A, Cordier C, Conley ME, et al. Human blood IgM "memory" B cells are circulating splenic marginal zone B cells harboring a prediversified immunoglobulin repertoire. Blood. 2004;104(12):3647-54.
22. Berkowska MA, Driessen GJ, Bikos V, Grosserichter-Wagener C, Stamatopoulos K, Cerutti A, et al. Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways. Blood. 2011.
23. Kalina T, Flores-Montero J, van der Velden VH, Martin-Ayuso M, Bottcher S, Ritgen M, et al. EuroFlow standardization of flow cytometer instrument settings and immunophenotyping protocols. Leukemia. 2012;26(9):1986-2010.
24. Berkowska MA, Heeringa JJ, Hajdarbegovic E, van der Burg M, Thio HB, van Hagen PM, et al. Human IgE(+) B cells are derived from T cell-dependent and T cell-independent pathways. J Allergy Clin Immunol. 2014;134(3):688-97 e6.
25. van Zelm MC, Szczepanski T, van der Burg M, van Dongen JJ. Replication history of B lymphocytes reveals homeostatic proliferation and extensive antigen-induced B cell expansion. J Exp Med. 2007;204(3):645-55.
26. Brandtzaeg P, Farstad IN, Johansen FE, Morton HC, Norderhaug IN, Yamanaka T. The B-cell system of human mucosae and exocrine glands. Immunol Rev. 1999;171:45-87.
27. Crabbe PA, Carbonara AO, Heremans JF. The Normal Human Intestinal Mucosa as a Major Source of Plasma Cells Containing Gamma-a-Immunoglobulin. Laboratory investigation; a journal of technical methods and pathology. 1965;14:235-48.
28. Yaari G, Uduman M, Kleinstein SH. Quantifying selection in high-throughput Immunoglobulin sequencing data sets. Nucleic Acids Res. 2012;40(17):e134.
29. Uduman M, Yaari G, Hershberg U, Stern JA, Shlomchik MJ, Kleinstein SH. Detecting selection in immunoglobulin sequences. Nucleic Acids Res. 2011;39(Web Server issue):W499-504.
30. Berkowska MA, Grosserichter-Wagener C, Adriaansen HJ, de Ridder D, Mirani-Oostdijk KP, Agteresch HJ, et al. Persistent polyclonal B-cell lymphocytosis: extensively proliferated CD27+IgM+IgD+ memory B cells with a distinctive immunophenotype. Leukemia. 2014;28(7):1560-4.
31. van Zelm MC, Bartol SJ, Driessen GJ, Mascart F, Reisli I, Franco JL, et al. Human CD19 and CD40L deficiencies impair antibody selection and differentially affect somatic hypermutation. J Allergy Clin Immunol. 2014;134(1):135-44.
32. Jackson KJ, Wang Y, Collins AM. Human immunoglobulin classes and subclasses show variability in VDJ gene mutation levels. Immunol Cell Biol. 2014;92(8):729-33.
33. van Zelm MC. B cells take their time: sequential IgG class switching over the course of an immune response? Immunol Cell Biol. 2014;92(8):645-6.
34. Di Sabatino A, Rosado MM, Cazzola P, Biancheri P, Tinozzi FP, Laera MR, et al. Splenic function and IgM-memory B cells in Crohn's disease patients treated with infliximab. Inflammatory bowel diseases. 2008;14(5):591-6.
35. Janssen CE, Rose CD, De Hertogh G, Martin TM, Bader Meunier B, Cimaz R, et al. Morphologic and immunohistochemical characterization of granulomas in the nucleotide oligomerization domain 2-related disorders Blau syndrome and Crohn disease. J Allergy Clin Immunol. 2012;129(4):1076-84.

36. Sweiss NJ, Lower EE, Mirsaeidi M, Dudek S, Garcia JG, Perkins D, et al. Rituximab in the treatment of refractory pulmonary sarcoidosis. Eur Respir J. 2014;43(5):1525-8.
37. Chen H, Liao D, Holl TM, Snowden P, Ueda Y, Kelsoe G. Genetic regulation of pristane-induced oil granuloma responses. International journal of experimental pathology. 2010;91(5):472-83.
38. Hermaszewski RA, Webster AD. Primary hypogammaglobulinaemia: a survey of clinical manifestations and complications. The Quarterly journal of medicine. 1993;86(1):31-42.
39. Blombery P, Prince HM, Levinson M, Pianko S, Maxwell E, Bhathal P. Rituximab-induced immunodysregulatory ileocolitis in a patient with follicular lymphoma. J Clin Oncol. 2011;29(5):e110-2.
40. Papadakis KA, Rosenbloom B, Targan SR. Anti-CD2O chimeric monoclonal antibody (rituximab) treatment of immune-mediated thrombocytopenia associated with Crohn's disease. Gastroenterology. 2003;124(2):583.
41. Rogosnitzky M, Danks R, Holt D. Intravenous immunoglobulin for the treatment of Crohn's disease. Autoimmun Rev. 2012;12(2):275-80.
42. Nishida A, Lau CW, Mizoguchi E, Mizoguchi A. Regulatory B cells in mouse models of intestinal inflammation. Methods Mol Biol. 2014;1190:227-41.
43. Sims GP, Ettinger R, Shirota Y, Yarboro CH, Illei GG, Lipsky PE. Identification and characterization of circulating human transitional B cells. Blood. 2005;105(11):4390-8.
44. Isnardi I, Ng YS, Menard L, Meyers G, Saadoun D, Srdanovic I, et al. Complement receptor 2/CD21- human naive B cells contain mostly autoreactive unresponsive clones. Blood. 2010;115(24):5026-36.
45. Moir S, Malaspina A, Ogwaro KM, Donoghue ET, Hallahan CW, Ehler LA, et al. HIV-1 induces phenotypic and functional perturbations of B cells in chronically infected individuals. Proc Natl Acad Sci U S A. 2001;98(18):10362-7.
46. Wehr C, Eibel H, Masilamani M, Illges H, Schlesier M, Peter HH, et al. A new CD21low B cell population in the peripheral blood of patients with SLE. Clin Immunol. 2004;113(2):161-71.
47. Verstegen RH, Driessen GJ, Bartol SJ, van Noesel CJ, Boon L, van der Burg M, et al. Defective B-cell memory in patients with Down syndrome. J Allergy Clin Immunol. 2014.
48. Rakhmanov M, Gutenberger S, Keller B, Schlesier M, Peter HH, Warnatz K. CD21low B cells in common variable immunodeficiency do not show defects in receptor editing, but resemble tissue-like memory B cells. Blood. 2010;116(18):3682-3.
49. Li Z, Vermeire S, Bullens D, Ferrante M, Van Steen K, Noman M, et al. Anti-Tumor Necrosis Factor Therapy Restores Peripheral Blood B-cell Subsets and CD40 Expression in Inflammatory Bowel Diseases. Inflammatory bowel diseases. 2015;21(12):2787-96.
50. Neurath MF, Travis SP. Mucosal healing in inflammatory bowel diseases: a systematic review. Gut. 2012;61(11):1619-35.
51. Berkowska MA, Schickel JN, Grosserichter-Wagener C, de Ridder D, Ng YS, van Dongen JJ, et al. Circulating Human CD27-IgA+ Memory B Cells Recognize Bacteria with Polyreactive Igs. J Immunol. 2015;195(4):1417-26.

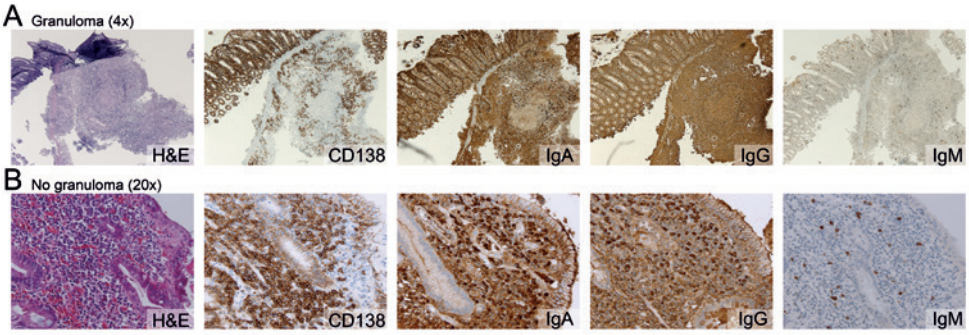
SUPPLEMENTAL TABLE. Targeting and selection of individual mutations in rearranged *IGHV*

	Natural effector				**IgA Memory**				**IgG Memory**			
	Control (n=120)		**Crohn (n=51)**		**Control (n=112)**		**Crohn (n=144)**		**Control (n=129)**		**Crohn (n=123)**	
Mutated rear. (%)	100/120	(83.3)	39/51	(76.5)	111/112	(99.1)	142/144	(98.6)	126/129	(97.7)	121/123	(98.4)
Transitions (%)	504/909	(55.4)	123/224	(54.9)	997/1850	(53.9)	1779/3503	(50.8)	1241/2438	(50.9)	1313/2653	(49.5)
Transversions (%)	405/909	(44.6)	101/224	(45.1)	853/1850	(46.1)	1724/3503	(49.2)	1197/2438	(49.1)	1340/2653	(50.5)
Transitions at C·G (%)	298/543	(54.9)	64/129	(49.6)	563/1076	(52.3)	991/2000	(49.6)	743/1432	(51.9)	777/1552	(50.1)
Targeting of C·G (%)	543/909	(59.7)	129/224	(57.6)	1076/1850	(57.7)	2000/3503	(57.1)	1432/2438	(58.7)	1552/2653	(58.3)
RGYW (%)	244.1/909	(26.9)	57.1/224	(25.5)	483.3/1850	(26.1)	**774.6/3503**	**(22.1)***	613.7/2438	(25.2)	**584.8/2653**	**(22.0)***
WRCY (%)	132/909	(14.5)	28.3/224	(12.6)	264.6/1850	(14.3)	536.8/3503	(15.3)	351.7/2438	(14.4)	372.5/2653	(14.0)
WA (%)	131.7/909	(14.5)	29.5/224	(13.1)	252.3/1850	(13.6)	449.6/3503	(12.8)	303.7/2438	(12.5)	**164.3/2653**	**(13.7)***
TW (%)	45.2/909	(5.0)	15.4/224	(6.9)	151.9/1850	(8.2)	243/3503	(6.9)	159.0/2438	(6.5)	171.3/2653	(6.5)
FR (R/S)	379/212	(1.8)	**69/63**	**(1.5)***	719/460	(1.6)	1519/954	(1.6)	1065/618	(1.7)	1150/697	(1.6)
CDR (R/S)	259/59	(4.4)	51/14	(3.6)	535/134	(4.0)	780/238	(3.3)	596/159	(3.7)	645/158	(4.1)

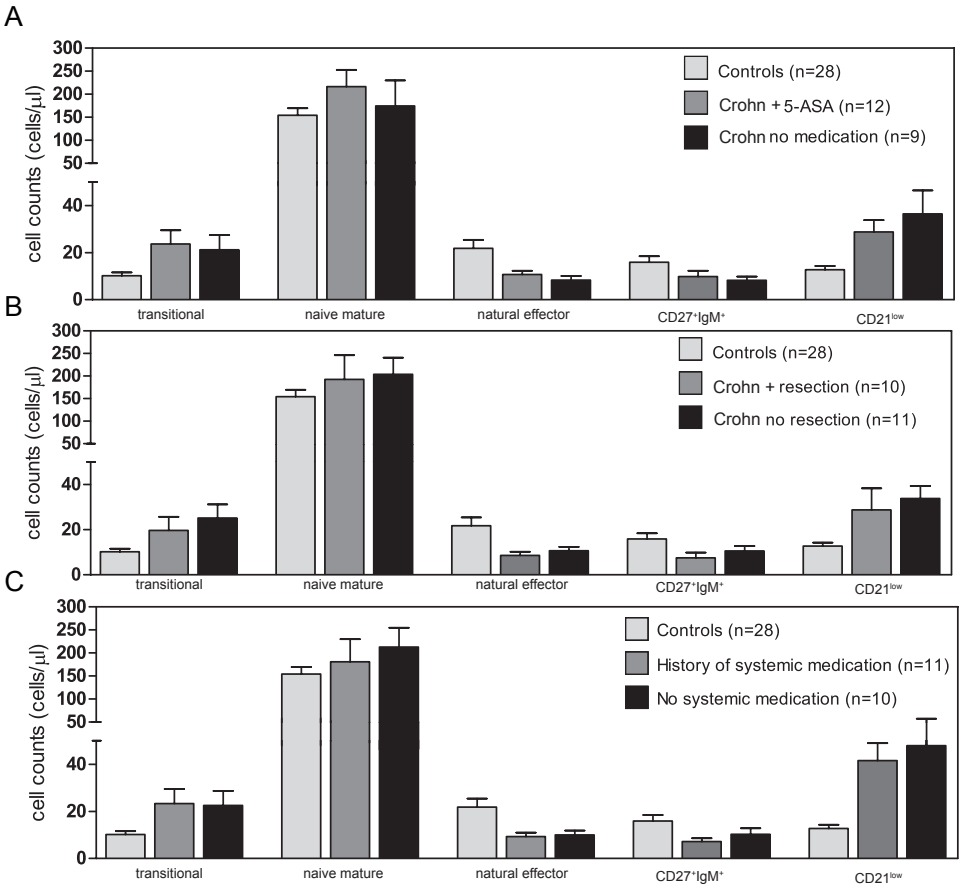
FR denotes framework region; CDR, complementarity determining region; R/S is the ratio between replacement (R) and silent mutations (S); the numbers of analyzed sequences are indicated in brackets next to the population name. All analyses were performed with the JOINSOLVER™ program and the differences between controls and patients were analyzed with the $\chi 2$ test. *, $P<0.05$



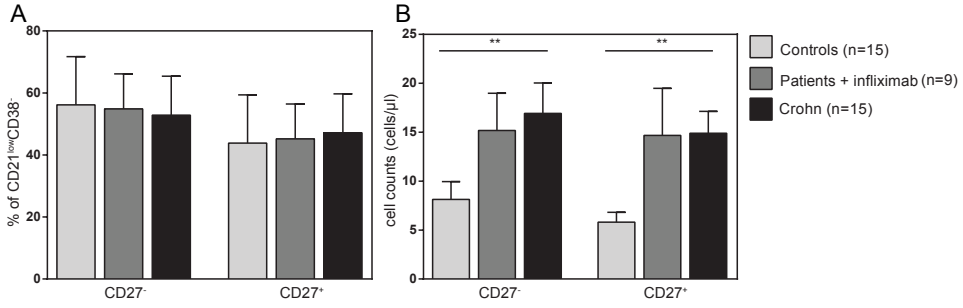
SUPPLEMENTAL FIGURE 1. Flowcytometric gating strategy for the described B-cell subsets in a representative healthy control **(A)** and a patient with Crohn's disease **(B).** B cells were gated within CD19 gate with two naive subsets; transitional cells and naive mature cells, six memory cells, plasma cells and CD38^{dim} C21^{low} cells. Unswitched memory B cells were separated into natural effector (CD27^{+}IgM^{+}IgD^{+}) and IgM-only cells (CD27^{+}IgM^{+}IgD^{-}). IgA and IgG switched memory B cells were further separated into CD27^{-} and CD27^{+} subsets.



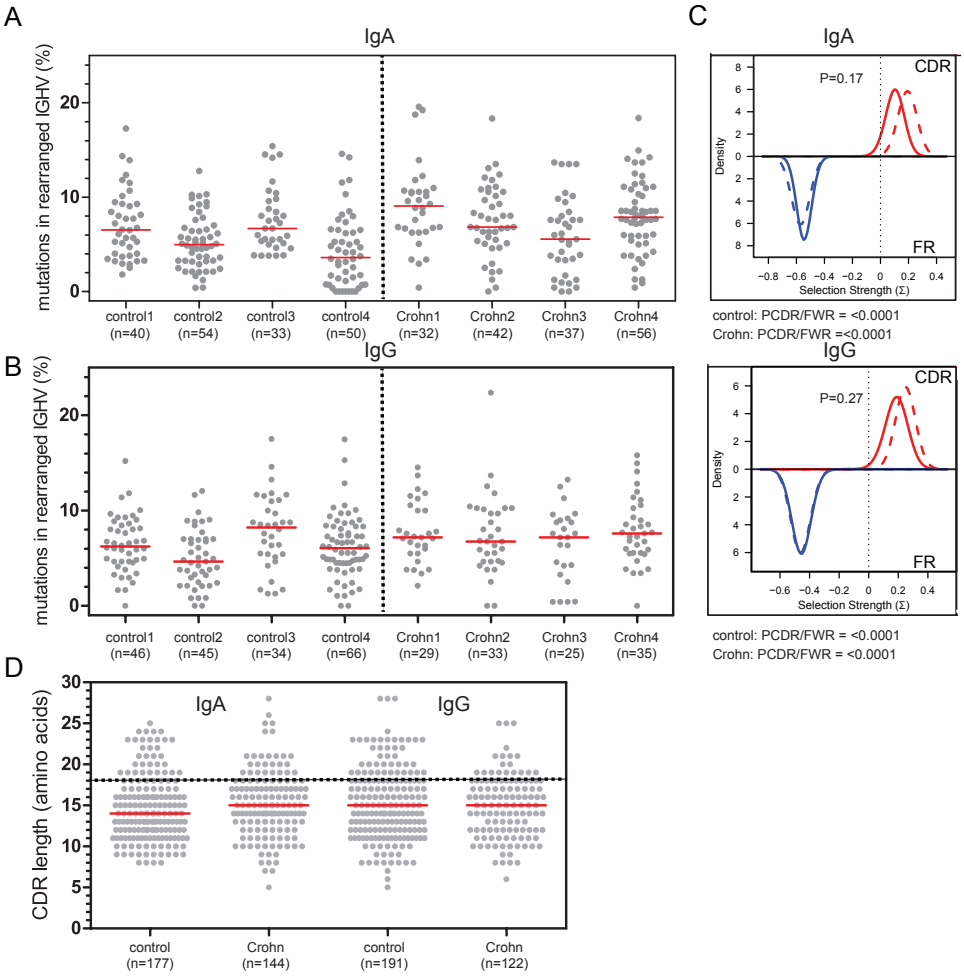
SUPPLEMENTAL FIGURE 2. Immunohistological analysis of plasma cells in sections with **(A)** and without granulomas **(B)** in colon biopsies of two patients with Crohn's disease. Both tissues show presence of CD138^{+} plasma cells, with the majority producing IgA, to a lesser extent IgG and low frequencies IgM.



SUPPLEMENTAL FIGURE 3. Blood B-cell compartments in **(A)** patients with a history of resection, **(B)** patients under treatment with 5-ASA medication, and **(C)** patients with a history of systemic immunosuppressive medication. Bars represent mean values ±SEM. No significant differences were found for any subset between Crohn's patients with or with the indicated mode of treatment (Mann-Whitney U test).



SUPPLEMENTAL FIGURE 4. CD21^{low} population in controls, patients on infliximab and Crohn's disease patients without systemic treatment. **A,** Relative distribution of CD27^{-} and CD27^{+} cells within the CD21^{low} compartment. **B,** Absolute cell counts of CD27^{-} and CD27^{+} cells within CD21^{low} compartment. Bars represent mean values ±SEM.



SUPPLEMENTAL FIGURE 5. Somatic hypermutation analysis of IgM, IgA and IgG B cells. Somatic hypermutation levels in IGHV genes of rearranged IgA **(A)** and IgG **(B)** transcripts of four patients with Crohn's disease and four healthy controls. Grey dots represent unique sequences; red lines represent median values. **C,** Selection for replacement mutation in IGHV-CDR (red line) and IGHV-FR regions (blue lines) as determined with the BASELINe program (28, 29). Solid lines represent patients; dashed lines represent healthy controls. Selection Strengths >0 indicate positive selection. **D**, IGH-CDR3 size distributions. All individual sizes are indicated as grey dots, red lines representing median values. The dashed line represents median values for centroblasts and centrocytes from controls (22).


Flinders Street
9

CHAPTER 3

IMPROVED DETECTION OF GRANULOMAS BY STAINING FOR B-CELLS: IMPLICATIONS FOR DIFFERENTIAL DIAGNOSIS BETWEEN ULCERATIVE COLITIS AND CROHN'S DISEASE

W.M.C. Timmermans [1,2], K.H. Lam [3], F.J. van Kemenade [3], PLA van Daele [1,2], P.M. van Hagen[1,2], **J.A.M. van Laar** [1,2], **M.C. van Zelm** [2,4]

[1] Department of Internal Medicine, Erasmus MC, Rotterdam, The Netherlands
[2] Department of Immunology, Erasmus MC, Rotterdam, The Netherlands
[3] Department of Pathology, Erasmus MC, Rotterdam, The Netherlands
[5] Department of Immunology and Pathology, Monash University and Alfred Hospital, Melbourne, VIC, Australia
Authors in bold designate shared last authorship

Submitted

ABSTRACT

Background and aims

Histological identification of granulomas is a key finding in rendering a definitive diagnosis of Crohn's disease. However, granulomas are only found in 9-29% of cases in H&E stained tissue. As a result, differential diagnosis between Crohn's disease and ulcerative colitis often relies on a combination of clinical and histological features, which are suboptimal as illustrated by a change of diagnosis of up to 10% of patients during clinical follow-up.

Methods

Based on earlier reported observations that granulomas are surrounded by B cells, we retrospectively studied whether staining for B-cells could improve the detection of Crohn's disease in patients with inflammatory bowel disease (IBD).

Results

CD20 stainings on 44 Crohn's disease biopsies increased granuloma detection 1.8 fold from 20% to 36% of cases. Importantly, in 3/39 subtotal colectomy samples of IBD patients, CD20 staining retrospectively supported a diagnosis of Crohn's disease instead of ulcerative colitis. Furthermore, dense B-cell infiltrates in biopsies of patients with Crohn's disease were objective indicators for the presence of granulomas.

Conclusions

CD20 staining improves sensitivity for detection of granulomas in Crohn's disease can be straightforwardly implemented in the diagnostic work-up of IBD. Early and accurate differentiation of Crohn's disease and ulcerative colitis can improve treatment outcomes, especially after surgery.

INTRODUCTION

A correct diagnosis is still challenging in patients with inflammatory bowel disease (IBD). Eventually up to 9% of patients originally diagnosed with ulcerative colitis (UC) are later re-diagnosed as Crohn's disease.[1] Yet, it can be important to distinguish between UC and Crohn's disease for optimal disease management and reliable research results.[2,3] Specifically, ileal-anal anastomosis is a widely accepted surgical procedure in patients with UC, while it is usually only recommended in a highly selected group of patients with Crohn's disease, for example with isolated colitis without peri-anal disease, due to an increased risk of a number of complications including pouch failure.[2,4] In spite of recent studies including genomic and serologic biomarkers, the diagnosis IBD is still made through combining clinical, endoscopic and histopathologic features. The presence of epithelioid granulomas in IBD is the single most defining histological finding to support the clinical diagnosis of Crohn's disease.[5,6] However, these collections of epithelioid macrophages are often small or undetectable in the tissue biopsies.[7-9] Granulomatous inflammation in both Crohn's disease and sarcoidosis is associated with large B-cell infiltrates.[10-12] As B-cells were usually not examined in non-granulomatous tissue,[10] we hypothesized that staining of tissue biopsies with a B-cell marker (CD20) in addition to the standard hematoxylin and eosin (H&E) stain might improve detection of granulomas as compared to the routine pathology analysis using only H&E stain. In this study, we tested the value of additional CD20 staining through: 1) retrospective analysis of granuloma detection in biopsies of patients with a clinical diagnosis of Crohn's disease; 2) retrospective analysis in IBD samples of subtotal colectomies. Furthermore, we tested whether the presence of more B cells were indicative of an increased rate of granuloma formation.

METHODS

STUDY COHORTS

In this study, we performed retrospective analysis in two patient cohorts. The first consists of endoscopy biopsies from 50 randomly selected patients who were clinically diagnosed with Crohn's disease from 2000 to 2005. Tissue blocks were retrieved from the pathology database containing diagnostic biopsies of colon or ileum obtained with colonoscopy. Inclusion criteria were: histologically proven Crohn's disease, history of Crohn's disease or a high index of suspicion for Crohn's disease by the gastroenterologist when performing the endoscopy. Biopsies were excluded if there were only taken for colon cancer surveillance or if the tissue samples were from patients from other hospitals analyzed for a second opinion.

The second cohort consists of all 47 patients who had undergone subtotal colectomies with an inflammatory character between 2000 and 2005. The same exclusion criteria were applied as for the biopsies above.

All tissue samples were obtained from the archives of the Department of Pathology of the Erasmus MC. Ethical approval was waived since in The Netherlands retrospective cross-sectional studies are not subject to consent. Still, this study was conducted in accordance with the Code of Conduct of the Federation of Dutch Medical Scientific Societies (FDMSS) for responsible use of human tissue in medical research.

HISTOLOGICAL ANALYSIS

Tissue from the biopsies and resection specimens were fixed in 10% neutrally buffered formalin, processed and embedded in paraffin blocks according to standard pathology laboratory procedures. Slides of 2µm were cut from the paraffin blocks, mounted on glass slides and stained with H&E on the Symphony platform (Ventana, Tucson, USA). Immunohistochemistry was performed on 4 µm sections using the same monoclonal antibody against CD20 (L26, Dako Cytomation, Glostrup, Denmark) on the Benchmark Ultra platform (Ventana, Tucson, USA) that we had previously used to define the presence of B cells in biopsies of patients with Crohn's disease, sarcoidosis and Behçet's disease. [10,11,13]

To minimize selection and observer bias, all included biopsy samples were blinded to the researchers, making them unaware of the actual diagnosis of the resections and the initially reported presence of granulomas. All biopsies were independently scored for non-crypt-associated granulomas characteristic for Crohn's disease by an experienced pathologist (KL) together with an investigator (WT). One slide per patient per staining was observed. A qualitative method of granuloma scoring was used. Biopsies were scored for either the presence or absence of granulomatous inflammation ranging from one small granuloma to many large granulomas. All crypt-associated granulomas were excluded because they are suggestive of ulcerative colitis. Observer bias was further reduced by randomly reevaluating the slides with the following sequential approach: 1) all H&E slides; 2) all CD20 slides; 3) linking results of H&E and CD20 and in case of a different outcome both were repeated together; 4) comparing the combined result of H&E with CD20 on the presence or absence of granulomas with the original H&E reports for statistical analysis. The presence of B cells was scored semi-quantitatively. The results were distributed over four groups: 'CD20-' (<10 B cells), 'CD20+' (< 3 foci with <50 cells), 'CD20++' (>3 foci with >50 cells), CD20+++ (>5 foci with >100 cells).

The resection samples were analyzed using a similar approach. As surgical resections have a large number of tissue sections, appropriate sections had to be selected first. All sections were screened using the original H&E slides, and the section containing the

densest lymphoid infiltrates was selected for analysis. Sections of the included samples were then cut, stained and analyzed as described above. Finally, the established diagnosis, the follow-up on surgical complications or whether the diagnosis changed in a later stage were retrieved from the medical record of the patients. There was no need to semi-quantitatively score B cells, as was performed in the biopsies, because all resection samples contained large B-cell infiltrates.

STATISTICAL ANALYSIS

A sample size analysis was performed to test the hypothesis whether CD20 addition would lead to a higher prevalence of granulomas in biopsies of Crohn's disease patients. Given the prevalence of granulomas in literature, sample size was calculated with 30% granuloma with standard H&E staining that would potentially increase to 60% with H&E and CD20 based on preliminary results of a small pilot study. With alpha 0.05, the sample size provided was 42 samples. Sensitivity and specificity were calculated by comparing the rate of granulomas found in the original histological report by H&E and with re-analysis with H&E to the rate found in our combined H&E with CD20 staining. In this calculation the reevaluation with the combination of H&E and CD20 staining was considered as golden standard. Differences in granuloma rates between H&E re-analysis as compared to the combined CD20 and H&E analysis were evaluated using the $\chi 2$ test (GraphPad Prism version 7). The $\chi 2$ test was also applied to test differences in the degrees of B-cell densities (CD20-, CD20+, CD20++, CD20+++) between granuloma positive and negative biopsies. A P-value <0.05 was considered statistically significant.

RESULTS

CD20 STAINING INCREASES THE DETECTION OF GRANULOMAS IN CROHN'S DISEASE BIOPSIES

From the initially selected 50 biopsies, insufficient material was left for this study, resulting in inclusion of 44 biopsies. Of these 44 biopsies from patients with Crohn's disease, 6 (14%) were documented to contain granulomas. H&E reanalysis alone also resulted in a higher number of cases with a certain presence of granulomas (9/44; 20%). Two additional cases showed a potential presence of granulomas. Subsequently, independent analysis of these 44 biopsies with combined H&E and CD20 stained slides resulted in a significantly higher detection of granulomas than H&E alone: 16/44 (36%) biopsies ($p=0.01$; $\chi 2$ test). These concerned the same 9 as detected with H&E alone, and the additional 7 were later confirmed in the H&E staining by visual support of CD20

(Figure 1). Of the 6 granulomas reported in the original files, two could not be detected even with CD20 staining due to sampling error. When considering the combination of CD20 and H&E as the new golden standard, the original sensitivity and specificity for the original H&E staining were 25% (4/16) and 93% (26/28), respectively (Table 1) and for the reanalyzed H&E combined with CD20 staining was 56% (9/16) and 100% (28/28) respectively.

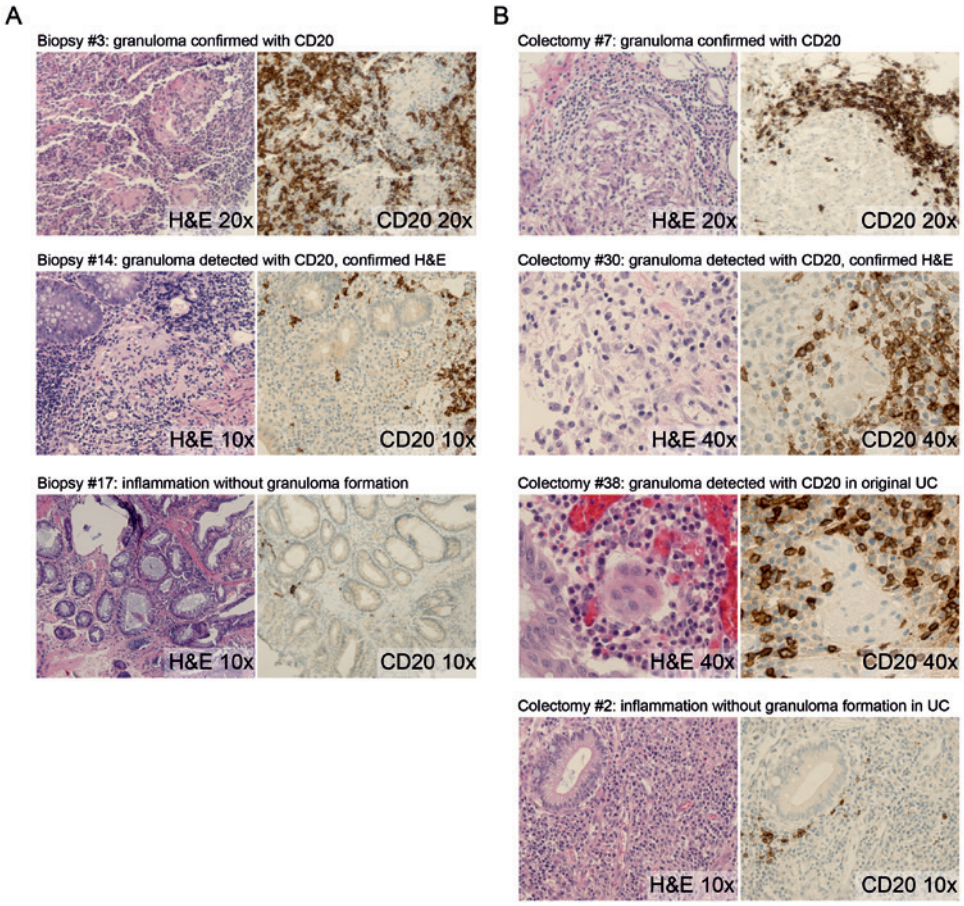


FIGURE 1. B cells visualize the presence of granulomas. (A) Representative images of biopsies from patients with Crohn's disease. (B) Images of samples of subtotal colectomy in patients with IBD.

TABLE 1. Retrospective analysis of granulomas with combined H&E and CD20 stainings in biopsy and colectomy samples

	Outcome H&E + CD20 analysis		
Original H&E	**Positive**	**Negative**	**Total**
Biopsy (n=44)			
positive	4	2	6 (14%)
negative	12	26	38
Total	16 (36%)	28	44
	Outcome H&E + CD20 analysis		
Re-analysis H&E	**Positive**	**Negative**	**Total**
Biopsy (n=44)			
positive	9	0	9 (20%)
negative	7	28	35
Total	16 (36%)	28	44
Colectomy (n=39)			
positive	8	0	8 (21%)
negative	5	26	31
Total	13 (33%)	26	39

RETROSPECTIVE IDENTIFICATION OF GRANULOMAS IN COLECTOMY TISSUE IN PATIENTS PREVIOUSLY DIAGNOSED WITH ULCERATIVE COLITIS

To further test the CD20 hypothesis we performed a similar analyses as above for 39 samples of subtotal colectomies in IBD patients. The original pathology files reported 17/39 (44%) specimens with a histopathological diagnosis of UC and 17 (44%) with Crohn's disease, of which 8 (47%) contained granulomas. Four patients (10%) were classified as indeterminate colitis while one patient appeared to have an infectious cause. Reanalysis with H&E of Crohn's disease resection samples confirmed the presence of granulomas in the 8 initially defined samples, without detection of these in additional cases. Combined H&E and CD20 analysis resulted in the detection of one additional granuloma-positive case in the Crohn's disease group. Furthermore, the combined analysis revealed granulomas in 4 cases of the non-Crohn's disease group, 3 of which (18%) were originally diagnosed with UC. These 3 patients, in contrast to the other UC patients, experienced a complicated disease course after surgery, fitting better with Crohn's disease than UC. One patient developed a peri-anal fistula two years after surgery and pouchitis six years after surgery. The second patient had a proctitis six months after surgery treated with a proctectomy and she developed a vaginal fistula with abdominal fluid pockets two years after surgery. The third patient had ileal pouch-anal anastomosis reconstructive surgery with the development of pouchitis two years

after surgery with focal, patchy inflammation seen during histopathological testing. This patient developed sacroileitis years later with a good response to infliximab. In all three cases, treating clinicians favored the diagnosis Crohn's disease years after surgery due to the course of disease. No granulomas were detected in the 4 indeterminate patients, while during follow up one patient was classified as UC, one as indeterminate colitis and two as Crohn's disease.

DENSE B-CELL INFILTRATES IN INTESTINAL BIOPSIES ARE INDICATIVE OF GRANULOMA PRESENCE

To study whether the presence and densities of CD20+ B cells in histopathological slides of biopsies were indicative of the presence of granulomas, we semi-quantitatively scored the numbers of B cells and defined 4 groups: 'CD20-' (<10 B cells), 'CD20+' (< 3 foci with <50 cells), 'CD20++' (>3 foci with >50 cells), CD20+++ (>5 foci with >100 cells). In all 5 cases without B cells, no granulomas were detected. Granulomas were detected in 2/13 (15%) and 2/7 (29%) cases scored as CD20+ and CD20++ respectively. Finally, 12/16 (75%) cases in the CD20+++ group contained granulomas. Importantly, when split into groups based on presence of granulomas (Figure 2), it was found that in significantly more granuloma positive slides large B-cell infiltrates were found (p=0.01; $\chi 2$ test).

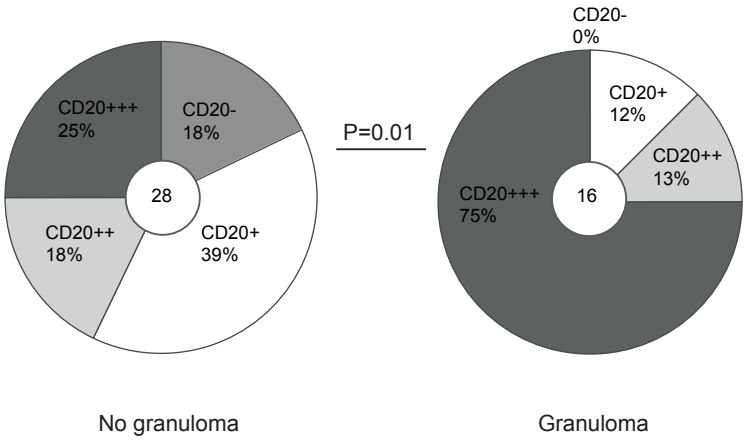


FIGURE 2. Relative densities of B cells in biopsy samples with and without granulomas. Biopsies were categorized based on B-cell densities (see main text). Statistical analysis was performed with $\chi 2$ test to analyze differences in distribution of samples over the 4 categories. Numbers of analyzed samples are depicted in centers of the pie charts.

DISCUSSION

In this study, we demonstrate that the combination of H&E with CD20 staining increased the detection of granulomas in biopsies of patients with Crohn's disease with 1.8 fold compared to the original approach with optimal H&E staining. These results suggest that additional CD20 staining may increase the sensitivity of IBD biopsies.

The reported granuloma rate reported in the original H&E of 14% is in the low-normal range compared to literature, suggesting that our randomized samples are representative of biopsies from patients with Crohn's disease.[5,6,14-16] Reevaluation with H&E staining increases the yield with 1.5 fold. However, this reevaluation occurs in an optimal setting using two investigators and is therefore time-consuming and not representative of daily practice. Compared to the setting as representative for the daily practice, addition with CD20 staining increases the detection of granulomas 1.8 fold. In this study, only one slide per patient has been evaluated. The number of detected granulomas might be higher if more sections were scored, especially in the surgical resection specimens as is often done in clinical practice.

The relative high prevalence of reported granulomas (47%) in the cohort of Crohn's disease patients can be explained by the high availability of larger tissue specimens. Complete specimen analyses reduces sampling error and enables evaluation of transmural inflammation. This higher prevalence is often described in literature in surgical cohorts and another explanation of the higher granuloma prevalence is the reported association of more aggressive disease resulting in a need of surgery. [15,17,18] Therefore, granulomas identified in surgical specimen can support a change of diagnosis in patients previously diagnosed with ulcerative colitis. In this study this was demonstrated as the combination of non-crypt-associated granulomas together with a complicated disease course with fistulas and pouchitis years after surgery retrospectively favored Crohn's disease. An improved accuracy in the distinction between Crohn's disease and ulcerative colitis could be important when new therapeutics targeting different yet specific inflammatory pathways will become available for patients with IBD. For example, Mongersen (an oligonucleotide targeting Smad7) is currently evaluated for patients with Crohn's disease, whereas Janus kinase inhibitors are tested for UC.[19]
CD20 staining can be easily implemented, because it is relatively inexpensive, pathologists are familiar with the staining as it is standardly used in evaluation of hematological malignancies and the positive correlation between the number of B-cells and the probability to find additional granulomas, as demonstrated in this paper. In this report we have used CD20.[20] However any readily available antibody used to demonstrate a B-cell phenotype may also be used. Still, it will be important to have this new diagnostic approach externally validated, especially to ensure that it does not result in high false positivity and to research the additional benefit in clinical practice for diagnosing Crohn's disease.

In conclusion, this study shows that the addition of CD20 to routine H&E staining in biopsies of IBD patients increases sensitivity of granuloma detection. CD20 can easily be implemented in diagnostics, and contribute to a faster and more accurate diagnosis of Crohn's disease. This will be of increasing importance in a time when new biologicals will become available for patients with either Crohn's disease or ulcerative colitis.

ACKNOWLEDGEMENTS

The authors thank Dr. J.A. Stoop for his support with the immunohistochemistry of the tissue samples. The authors are indebted to prof.dr. C.J. van der Woude (Department of Gastroenterology, Erasmus MC, Rotterdam, The Netherlands) for help to design this study and inclusion of tissue samples. This study was performed in the framework of the Molecular Medicine Postgraduate School of the Erasmus MC.

REFERENCES

1. Moss AC, Cheifetz AS. How often is a diagnosis of ulcerative colitis changed to crohn's disease and vice versa? *Inflamm Bowel Dis* 2008;**14 Suppl 2**:S155-6.
2. Tontini GE, Vecchi M, Pastorelli L, Neurath MF, Neumann H. Differential diagnosis in inflammatory bowel disease colitis: State of the art and future perspectives. *World J Gastroenterol* 2015;**21**:21-46.
3. Silverberg MS, Daly MJ, Moskovitz DN*, et al.* Diagnostic misclassification reduces the ability to detect linkage in inflammatory bowel disease genetic studies. *Gut* 2001;**49**:773-6.
4. Chang S, Shen B, Remzi F. When not to pouch: Important considerations for patient selection for ileal pouch-anal anastomosis. *Gastroenterol Hepatol (N Y)* 2017;**13**:466-75.
5. Le Berre N, Heresbach D, Kerbaol M*, et al.* Histological discrimination of idiopathic inflammatory bowel disease from other types of colitis. *J Clin Pathol* 1995;**48**:749-53.
6. Seldenrijk CA, Morson BC, Meuwissen SG*, et al.* Histopathological evaluation of colonic mucosal biopsy specimens in chronic inflammatory bowel disease: Diagnostic implications. *Gut* 1991;**32**:1514-20.
7. Pulimood AB, Ramakrishna BS, Kurian G*, et al.* Endoscopic mucosal biopsies are useful in distinguishing granulomatous colitis due to crohn's disease from tuberculosis. *Gut* 1999;**45**:537-41.
8. Theodossi A, Spiegelhalter DJ, Jass J*, et al.* Observer variation and discriminatory value of biopsy features in inflammatory bowel disease. *Gut* 1994;**35**:961-8.
9. Nikolaus S, Schreiber S. Diagnostics of inflammatory bowel disease. *Gastroenterology* 2007;**133**:1670-89.
10. Timmermans WM, van Laar JA, van der Houwen TB*, et al.* B-cell dysregulation in crohn's disease is partially restored with infliximab therapy. *PLoS One* 2016;**11**:e0160103.
11. Kamphuis LS, van Zelm MC, Lam KH*, et al.* Perigranuloma localization and abnormal maturation of b cells: Emerging key players in sarcoidosis? *Am J Respir Crit Care Med* 2013;**187**:406-16.
12. Timmermans WM, van Laar JA, van Hagen PM, van Zelm MC. Immunopathogenesis of granulomas in chronic autoinflammatory diseases. *Clin Transl Immunology* 2016;**5**:e118.
13. van der Houwen TB, van Hagen PM, Timmermans WM*, et al.* Chronic signs of memory b cell activation in patients with behcet's disease are partially restored by anti-tumour necrosis factor treatment. *Rheumatology (Oxford)* 2017;**56**:134-44.
14. Freeman HJ. Granuloma-positive crohn's disease. *Can J Gastroenterol* 2007;**21**:583-7.
15. Heresbach D, Alexandre JL, Branger B*, et al.* Frequency and significance of granulomas in a cohort of incident cases of crohn's disease. *Gut* 2005;**54**:215-22.
16. Turner K, Genta RM, Lujan G, Robiou C, Sonnenberg A. Significance of the epithelioid granuloma in biopsies of crohn's colitis. *Inflamm Bowel Dis* 2014;**20**:2271-5.
17. Johnson CM, Hartman DJ, Ramos-Rivers C*, et al.* Epithelioid granulomas associate with increased severity and progression of crohn's disease, based on 6-year follow-up. *Clin Gastroenterol Hepatol* 2018;**16**:900-7 e1.
18. Kanneganti M, Dehghani B, Steinberg J*, et al.* Significance of granulomatous inflammation found on endoscopic biopsies or surgical resections on the severity of crohn's disease. *J Clin Gastroenterol* 2013;**47**:894.
19. Argollo M, Fiorino G, Hindryckx P, Peyrin-Biroulet L, Danese S. Novel therapeutic targets for inflammatory bowel disease. *J Autoimmun* 2017;**85**:103-16.
20. Wang HY, Zu Y. Diagnostic algorithm of common mature b-cell lymphomas by immunohistochemistry. *Arch Pathol Lab Med* 2017;**141**:1236-46.


Flinders Street
9

CHAPTER 4

BLOOD B- AND T-CELL KINETICS, SIL-2R, INFLIXIMAB TROUGH LEVELS AND ADA FORMATION INDICATE THERAPEUTIC SUCCESS OF INFLIXIMAB IN PATIENTS WITH SARCOIDOSIS

W.M.C. Timmermans[1,2], W.A. Dik[2], M.W.J. Schreurs[2], J.R. Miedema[3], M.S. Wijsenbeek[3], P.M. van Hagen[1,2], M.C. van Zelm [2,3]*, J.A.M. van Laar[1,2]*

[1] Department of Internal Medicine, Section of Clinical Immunology, Erasmus MC, Rotterdam, The Netherlands
[2] Department of Immunology, Laboratory Medical Immunology, Erasmus MC, Rotterdam, The Netherlands [3] Department of Pulmonology, Erasmus MC, Rotterdam, The Netherlands
[4] Department of Immunology and Pathology, Monash University and The Alfred Hospital, Melbourne, VIC, Australia

** shared senior authorship*

Manuscript submitted

ABSTRACT

Background and aims

Infliximab is used as third-line treatment in sarcoidosis with mixed therapeutic efficacy. Therefore, it would be beneficial to establish markers that predict potential treatment success. Patients with sarcoidosis have reduced memory B cells and CD4 helper T-cell subsets compared to healthy controls. In this study, we evaluated cellular and serological immunological markers in sarcoidosis patients before and during infliximab therapy.

Materials and Methods

The therapeutic response to infliximab was clinically evaluated in all 11 patients along with serum levels of sIL-2R, IgG, BAFF, and infliximab trough levels. Furthermore, extensive flowcytometric analysis of blood B and T-cell subsets was performed of all 11 patients before and during infliximab therapy (2 and 6 weeks, 8 months).

Results

Nine (82%) patients showed an objective clinical response. sIL-2R levels rapidly declined after the first infliximab infusion, whereas serum IgG and BAFF levels did not alter. IgM^+ memory B cell numbers increased in patients who clinically responded to infliximab therapy, as did the numbers of CD4, CD8 T cells, regulatory T cells, Th17 and Th17.1 T cells. Those who developed anti-drug antibodies (ADA), displayed lower levels of sIL-2R and Tregs with higher IgM-only B cells at baseline than responders to infliximab. Most patient had infliximab levels within therapeutic range and at months these levels correlated significantly with sIL-2R levels.

Conclusions

Our study confirms the potential of monitoring infliximab trough levels, sIL-2R and ADA as promising markers for therapeutic success in sarcoidosis patients. Moreover, infliximab therapy normalizes IgM+ memory B cells and Th subsets, demonstrating cellular immune effects and the potential for new laboratory markers of therapy success.

INTRODUCTION

Sarcoidosis is a chronic inflammatory disorder characterized by the presence of non-necrotizing granulomas affecting various organs including lungs, eyes, skin and the central nervous system.(1) The etiology is still unknown and the clinical presentation is highly heterogeneous, which relates to the organs involved. Fortunately, over 50% of patients will have a spontaneous remission within 3 years. Therefore, only patients with organ threatening sarcoidosis are treated with medication.(1, 2)

The pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α) exerts a pivotal role in both granuloma formation and maintenance by promoting infiltration of macrophages and lymphocytes.(3) Accordingly, in animal models of tuberculosis, granulomas appear disorganized in absence of TNF-α, and established granulomas are disrupted upon blocking of TNF signaling.(4, 5) Cultured alveolar macrophages from patients with sarcoidosis spontaneously produce more TNF-α, and higher levels of TNF-α are associated with progressive disease.(6, 7) Subsequently, in addition to first and second line therapy with corticosteroids and disease modifying drugs, TNF-α blockers have emerged as an option for third line treatment in sarcoidosis.(1)

Level 1 evidence for a beneficial effect of TNF-α blockers in sarcoidosis is restricted to one randomized controlled trial (RCT) with infliximab. It is thought that the selection of patients with stable, chronic disease including fibrosis has led to an unexpected modest response in FVC.(8) Therefore, concerns over its clinical significance in chronic pulmonary sarcoidosis have been raised. However, subsequent clinical trials and case series have shown significant beneficial effects in patients with extrapulmonary manifestations or in selected patients with severe and active disease.(9-12) Patients with severe or organ threatening disease are currently treated with infliximab after failing first and second line treatment. Especially patients with neuro, ocular, or cutaneous disease respond to infliximab in overall disease improvement ranging from 60% to 92%. (9-12) Furthermore, identification of potentially responding patients, timing of initiation and discontinuation of infliximab are challenging. In fact, most of our knowledge is derived from extensive experience with TNF-α blockers in Crohn's disease and rheumatoid arthritis.(13, 14) In these diseases infliximab trough levels correlate well with therapeutic efficacy and are therefore monitored to adapt for dose and treatment intensifications.(15, 16) In general, a target range of infliximab trough levels between 3 and 10 μg/mL is aimed to optimize therapeutic efficacy, minimize adverse events and to reduce costs as ineffective and supra therapeutic dosage can be prevented. When infliximab levels measured are <1 μg/mL further testing for antidrug antibodies (ADA) is recommended as ADA can form immune complexes with infliximab leading to very low trough levels and therapy failure.(17) Several other immunological monitoring tools have been proposed in the past years in sarcoidosis. Currently, serum soluble (s)IL-2R level is mostly used in daily practice and regarded as most sensitive to monitor disease activity.(18, 19)

Sarcoidosis patients typically display reduced CD4 T cell blood numbers due to accumulation of these cells in the affected organs.(20) Traditionally, sarcoidosis is regarded as a T-helper 1 (Th)1 driven disease, whereas more recently Th17.1 cells were identified as important cells contributing to the pro-inflammatory environment in sarcoidosis through production of IL-17 and IFN-γ.(21-23) Previously, we have demonstrated extensive B-cell infiltrates around granulomas, and reduced levels of blood IgM^+ and Ig class switched memory B cells in patients with sarcoidosis.(24)

In this study, we aim to study if abnormalities in peripheral blood B-cell and T-cell subsets normalize in sarcoidosis patients during infliximab treatment and can be used in addition to sIL-2R as early markers for therapy success.

MATERIALS AND METHODS

PATIENTS

Clinical data and blood samples were collected from 11 sarcoidosis patients who were scheduled to start with infliximab therapy based on clinical indications (Table 1). The eleven patients were recruited from the Departments of Internal Medicine and Pulmonary Medicine of the Erasmus MC. Blood samples were collected before first infliximab infusion (week 0), at week 2 and 6 and around month 8 within hours before infliximab infusion. Infliximab treatment was started on a standard induction schedule at week 0, 2 and 6. After 6 weeks, patients received infliximab treatment every 6 to 8 weeks depending on the individual needs. The therapeutic response of infliximab was monitored individually according to standard clinical care by the treating physician, and included blood tests, pulmonary function tests and imaging studies such as chest X-ray, CT-thorax and MRI of the brain. Participants were recruited after the clinical decision was made to start infliximab treatment, and clinical data and blood samples were collected after written informed consent was obtained. Inclusion in our study did not interfere with or alter decisions for treatment. In addition, blood samples from 18 healthy controls, consisting of 10 males and 8 females with a mean age of 50 years (range, 31-70 years) were obtained once after informed written consent was given. This study was approved by the Medical Ethics Committee of Erasmus MC (ethics approval number MEC-2014-044) and in accordance with the Declaration of Helsinki.

TABLE 1. Clinical characterization of patients with sarcoidosis starting with infliximab therapy.

Patient	Sex	Age (yr)	Ethnicity	Disease duration (yr)	Localization	PET/SRS	Previous medication	Indication IFX
1	F	66	Asian	12	Lung, eye, skin, joints, NS	positive	HCQ, MTX, HUM	Refractory uveitis under HUM
2	F	53	African	8	Lung, eye, parotis, joints, liver	positive	CC, AZA, HUM	Uveitis under HUM with ADA
3	F	68	African	5	Lung, LN, skin, joints, kidney	positive	CC, MTX	Refractory systemic disease under CC and MTX
4	F	45	Caucasian	1	Skin, joints, liver	positive	CC, MTX	Active, systemic disease with adverse event MTX
5	F	37	Caucasian	12	Lung, LN, skin, parotis	positive	CC, AZA, MTX	Refractory pulmonary disease during CC and AZA
6	F	40	African	3	Lung	ND	CC, MTX	Refractory pulmonary disease during MTX
7	F	40	Caucasian	3	Lung, LN	positive	CC, MTX	Refractory pulmonary disease during MTX
8	M	48	Caucasian	4	Lung	ND	CC, MTX	Active pulmonary disease during MTX
9	F	43	Caucasian	1.5	NS, LN	positive	CC, AZA	Refractory NS during AZA
10	F	47	Caucasian	1.5	NS, LN	ND	CC, AZA	Refractory NS during AZA AND CC
11	F	46	Caucasian	1	NS, LN	ND	CC, AZA	Serious liver test elevations during AZA

Definitions of abbreviations: F, female; M, male; yr, years; NS, neurosarcoidosis; LN, lymph nodes; PET, position emission tomography; SRS, somatostatin receptor scintigraphy; ND, not determined; HCQ, hydroxychloroquine; MTX, methotrexate; HUM, humira; CC, corticosteroids; AZA, azathioprine; ADA, anti-drug antibody

QUANTIFICATION OF IMMUNOLOGICAL MARKERS

Blood was collected in heparin tubes for plasma preparation and whole blood analysis. Plasma was obtained from all blood samples to measure soluble protein levels. Plasma IgG was measured by an immunoturbidimetric method (Hitachi Analyzer; Roche, Basel, Switzerland). In addition, sIL-2R levels were measured with enzyme immunoassay according to the manufacturer standards (human sCD25/sIL-2R ELISA kit; Diaclone SAS). ELISAs were performed to quantify levels of B-cell activating factor (BAFF; R&D Systems), and trough levels of infliximab (Sanquin, Amsterdam, The Netherlands). Infliximab-specific IgG was measured by an antigen binding test (Sanquin, Amsterdam, The Netherlands).

FLOWCYTOMETRIC IMMUNOPHENOTYPING OF BLOOD B- AND T-LYMPHOCYTES

Flowcytometric analysis was performed within 24 hours after blood collection on whole blood. Absolute counts of blood lymphocytes, $CD4^+$ and $CD8^+$ T cells and $CD19^+$ B cells were obtained with a diagnostic lyse-no-wash protocol. 10-Color flowcytometry was performed after red blood cell lysis to define B-cell subsets: transitional, naive mature, and six memory subsets, as described previously,(25) using monoclonal antibodies against CD27-BV421 (M-T271), CD21-BV711 (B-ly4), IgG-PE (G18-145), IgD-PE-CF594 (IA6-2; all from BD), IgM-BV510 (MHM-88), CD38-BV605 (HIT2), CD275-APC (2D2; all from Biolegend), IgA-PE (IS11-8E10, Miltenyi-Biotec), CD19-PE-Cy7 (J3-119) and CD24-APC-AF750 (ALB9; both Beckman Coulter). CD4 subsets were defined as follicular helper T(fh) cells, Tregs, Th1, Th2, Th17, Th17/IFNγ, using the following monoclonal antibodies; CD27-BV21 (M-T271), CD4-BV510 (OKT4), CD45RA-BV605 (HI100), CD25-BV421 (BC96), CXCR3-FITC (G025H7), CCR6/CD196-PerCP-Cy5.5 (G034E3), CCR4-PE-Cy7 (TG6/CCR4), CD127-APC (A019D5), CD28-PerCP-Cy5.5 (CD28.2; all from Biolegend), CCR7/CD197-PE-CF594 (150503), and CD8-APC-AF750 (SK1; all from BD), CD45RO-FITC (UCHL1; Exbio), CXCR5-APC (51505; R&D systems).(26) All samples were acquired on a 4-laser LSRFortessa (BD Biosciences, San Jose, California, US) with standardized instrument settings.(27)

MOLECULAR ANALYSIS OF IMMUNOGLOBULIN HEAVY CHAIN (*IGH*) GENE TRANSCRIPTS

IGHA and *IGHG* transcripts were amplified from PBMC cDNA of patients 1, 2 and 3 before infliximab treatment and around 8 months into treatment (n = 3). Data from healthy controls (n = 6) were obtained previously.(28) IGHV3 and IGHV4 leader primers and consensus Cα or Cγ reverse primers were used.(25) PCR products were cloned into the pGEM-T easy vector (Promega, Madison, WI) and prepared for sequencing on an ABIPRISM 3130XL (Applied Biosystems, Carlsbad, CA). Obtained sequences

were analyzed with IMGT database (http://www.imgt.org/IMGT_vquest/vquest) and Bayesian estimation of Antigen-driven SELectIoN (BASELINe; http://selection.med.yale.edu/baseline/).(29, 30) IgA and IgG receptor subclasses were determined using the IGH reference sequence (NG_001019).

STATISTICS

To determine differences between healthy controls and baseline patient data, the non-parametric Mann Whitney U test was used. To analyze pharmacodynamics from baseline until month 8, one way anova was used. When significant ($p<0.05$), this was specified by using a paired t-test between the various time points. As the aim was to identify lymphocytic markers in patients responding well to infliximab therapy, statistical analysis of T-and B-cell subsets was performed in patients with a consistent therapeutic response without formation of formation of anti-drug antibodies (ADA) during the eighth month study period. Spearman correlation test was used to confirm correlation between biomarkers and clinical data. Statistical analysis and Figures were prepared using GraphPad Prism Software.

RESULTS

CLINICAL CHARACTERIZATION OF PATIENTS

Eleven patients (10 female) with biopsy-proven sarcoidosis were included in this follow-up study with a mean age of 48 years (range, 37-68 years). The majority of patients had multi-organ involvement demonstrated with PET-CT or somatostatin receptor scintigraphy positive disease (Table 1). All patients had a history of immunosuppressive treatments since all had serious and active disease. Four patients (1-4) had a more systemic, active disease including uveitis at the time of infliximab start, while patients 5-8 had merely a pulmonary indication for infliximab therapy and patients 9-11 experienced severe neurological involvement. Most patients had concomitant use of corticosteroids, methotrexate or azathioprine which was tapered or stopped in some cases (Table 2). All patients started the induction schedule, receiving 300-500 mg (5mg/kg) infliximab at weeks 0, 2 and 6, and every subsequent 6 to 8 weeks as per decision of the treating medical specialist. Patients 1-8 were treated with Remicade, and patients 9-11 received the biosimilar Remsima, which has become the standard product in our center.

In 9/11 patients, a clinical response was objectified by ophthalmological examination, pulmonary function, Chest X-ray or MRI brain (Table 2 and Figure 1). Pulmonary function tests were consistently performed for patients 2, 5-8 prior to and during this study, and all five patients showed an improvement in forced vital capacity (FVC) with a mean or

12.4% (range 8-25%) of predicted FVC (Figure 1). Patient 4 initially showed improvement by chest CT, yet arthritis remained. This patient had a relapse associated with formation of ADA against infliximab after 16 months. Patient 6 initially showed a good response, yet she relapsed after 22 months with formation of ADA. Both patient 4 and 6 switched to adalimumab therapy because of failing on infliximab, with stable outcome for patient 4 and excellent response seen in patient 6. Patient 9 initially had a very good response with complete remission on brain MRI and remission of symptoms. However, the patient relapsed at the end of this study; the development of ADA occurred and increased presence of liver enzymes in serum (ASAT up to 133U/L; normal <31U/L, ALAT up to 217U/L; normal <34U/L) necessitated cessation of infliximab. Patient 11 did not show a therapeutic response, as evidenced by increased uptake of gadolinium on brain MRI. This patient developed ADA at month seven. Hence, infliximab was discontinued, the patient was started on methylprednisolone together with adalimumab, and this patient was excluded from the study.

Infliximab was generally well tolerated by the eleven patients in our study. Adverse events were restricted to pulmonary tract infections in patients 3, 4 and 7; and elevation of liver enzymes in patient 9, which normalized upon discontinuation of infliximab.

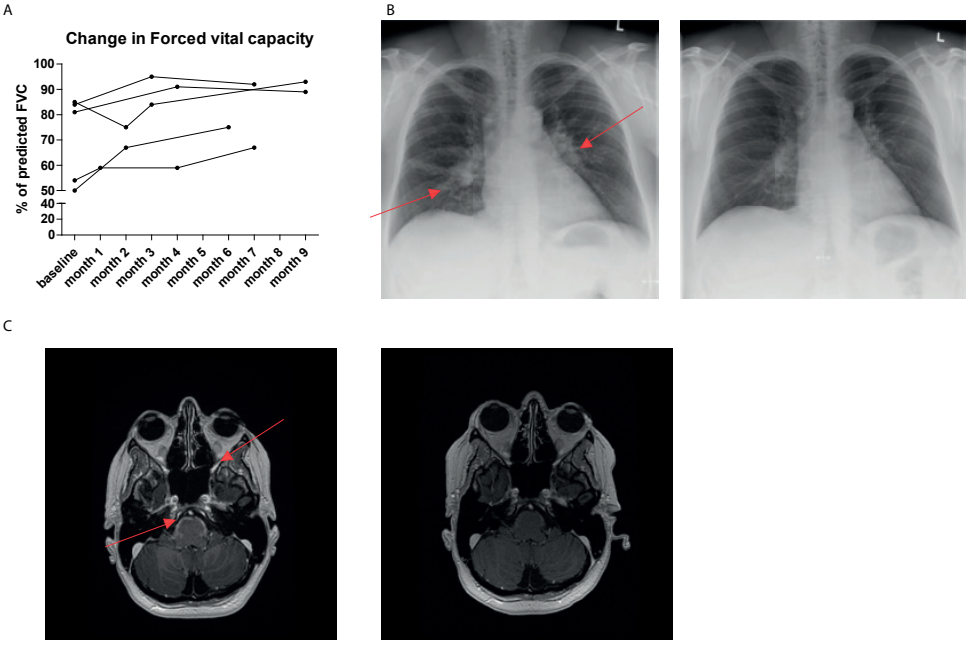


FIGURE 1. Clinical markers of follow-up of infliximab treatment. (A). Pulmonary function tests with % of predicted FVC before and during treatment with infliximab of five patients. Each dot represents an FVC value. **(B).** Chest X-ray of patient 7 at baseline (left) and after 7 months of successful treatment showing improvement with less prominent hilar lymph nodes and improved reticular opacities (right) **(C).** MRI Brain of patient 9 before therapy (left) and after 3 months of successful treatment (right) showing complete remission of leptomeningeal uptake.

TABLE 2. Follow-up results of patients receiving infliximab therapy.

Patient	Ifx dosage	Infusions doses at t=4	Medication t=0	Change in medication	Response	Follow-up during study	Infliximab	Adverse effects
1	400mg/8wk	6	MTX 15 mg	no	yes	Remission uveitis	Remicade	no
2	300mg/8wk	6	MTX 10, HCQ	no	yes	Remission uveitis, improved CT and LF	Remicade	no
3	300mg/8wk	6	CC 2.5 mg, MTX 20 mg	no	yes	Improved LF	Remicade	Respiratory tract infection
4*	400 mg/8wk	7	CC 5 mg	no	Mixed response	Improved CT, ongoing arthritis, late relapse with ADA	Remicade	Respiratory tract infection
5	400mg/6wk	9	CC 10 mg, AZA 150 mg	Stop CC, tapering AZA	yes	Improved LF	Remicade	no
6*	500mg/6wk	9	CC 5 mg, MTX 15 mg	Stop CC, tapering MTX	yes	Improved CT and LF, late relapse with ADA	Remicade	no
7	500mg/6wk	8	MTX 15 mg	no	yes	Improved Chest X-ray and LF	Remicade	Respiratory tract infection
8	400mg/6wk	8	MTX 10 mg	no	yes	Improved Chest X-ray and LF	Remicade	no
9**	300mg/8wk	5	CC 35 mg, AZA 125 mg	Stop CC and AZA	yes	MRI Brain complete remission at first, relapse during study with ADA	Remsima	Increased liver enzymes
10	500mg/6wk	6	CC 40mg, AZA 150mg	Tapering CC 10mg and AZA	yes	MRI Brain lesions improved significantly	Remsima	no
11**	400mg/6wk	N/A	no	no	no	MRI Brain relapse with ADA	Remsima	no

Pulmonary function FVC and DLCO presented in % expected. Definitions of abbreviations: ifx, infliximab; mg, milligram; wk, week; t, time-point of blood withdrawal; HCQ, hydroxychloroquine; MTX, methotrexaat; HUM, Humira; CC, corticosteroids; AZA, azathioprine; ADA, anti-drug antibody; LF, lung function; FVC, forced vital capacity; DLCO, diffusing CT, computed tomography; MRI, magnetic resonance imaging; N/A, not applicable. Patients * experienced relapse with formation of ADA. Patients ** relapsed during the study with formation of ADA.

DECLINE IN PLASMA SIL-2R BUT NOT IGG AND BAFF LEVELS FOLLOWING INFLIXIMAB TREATMENT

To study immunological success of infliximab therapy, we measured sIL-2R plasma levels in all patients prior to and during therapy. Plasma sIL-2R levels, but not IgG and BAFF, declined during infliximab therapy, and were inversely correlated to serum infliximab trough levels (Figure 2). At baseline, all but two patients, patients 9 and 11 without long-term response with ADA (Figure 1A**;** triangles), had elevated sIL2-R levels above the upper limit of 2,500 pg/mL (range between 1,365 and 20,900 pg/mL, Figure 2A). Following the first infusion of infliximab, sIL-2R levels significantly decreased (p=0.002) at week two with a long-term effect up to 8 months (p=0.049).

Only patients 1 and 6 had an IgG at baseline that was above the normal range (Figure 2B). In the current study the levels of IgG appeared to be rather stable within patients during infliximab therapy. Similarly, only 6/11 patients had BAFF plasma levels above the upper limit of healthy controls (range healthy controls 728-1295 pg/mL), and these levels did not change significantly during therapy (Figure 2C), yet BAFF levels did correlate significantly at baseline with sIL-2R levels (Figure 2F**;** p=0.05).

To study if the lack of response in clinical markers was related to suboptimal medication, infliximab trough levels were determined in all patients with the target. None of the 11 patients had infliximab levels above 10 µg/mL. At month 8, four patients had decreased infliximab trough levels (normal levels; 3-10 µg/ml). Despite this, only one patient (patient 9) also had ADA then. These were accompanied by infliximab levels in the undetectable range (0.06 µg/ml), whereas in the other three patients (1, 2 and 4) infliximab levels ranged from 1 to 2 µg/ml. Patients 1 and 2 displayed excellent long-term clinical responses without ADA formation, whereas patient 4 had a late relapse with ADA formation at 16 months, which is 8 months after the observation period of this study.

A significant inverse correlation was found at month 8 for infliximab trough levels and sIL-2R levels (Figure 2E**;** p=0.04).

FIGURE 2. Immunological markers and infliximab trough levels during infliximab treatment. Plasma levels of **(A)** sIL-2R, **(B)** IgG, **(C)** BAFF, and **(D)** infliximab at baseline, and week 2, week 6 and month 8 following start of infliximab treatment. Each dot represents a single measurement with lines connecting measurements of the same patient. Triangles represent data of the two patients who eventually developed ADA during this study, patient 9 and 11. The dashed line in panel A represents the upper limit of the normal range of sIL-2R (2500 pg/mL; upper limit of normal with the used test in our center). The upper and lower limits of the normal range of serum IgG are shown in panel B (7 and 16 g/l, resp), and well as the recommended target range of infliximab levels (between 3 and 10 µg/mL) in panel **D**.(17) The Mann-Whitney U test was used to statistically analyze differences between healthy controls and patients at baseline, whereas a paired T-test was used to examine therapy effects in patients; *, P<0.05; **, P<0.01. **E.** Regression analysis of infliximab trough levels and sIL-2R levels at month 8 using Spearman's rank correlation coefficient. **F.** Regression analysis of serum BAFF levels and sIL-2R levels at baseline using Spearman's rank correlation coefficient. →

A
plasma sIL-2R levels
pg/mL
25000
20000
15000
10000
5000
0
**
*
week 0
week 2
week 6
month 8
B
plasma IgG levels
g/l
25
20
15
10
5
0
week 0
week 2
week 6
month 8
C
plasma BAFF levels
pg/mL
5000
4000
3000
2000
1000
0
healthy controls
week 0
week 2
week 6
month 8
D
plasma trough infliximab levels
ug/mL
50
40
30
20
10
0
week 0
week 2
week 6
month 8
E
ifix ug/ml
10
8
6
4
2
0
p = 0.04
0
5000
10000
15000
sIL-2R (pg/ml)
F
BAFF (pg/mL)
4000
3000
2000
1000
0
p = 0.05
0
5000
10000
15000
20000
25000
sIL-2R (pg/ml)

TREG, TH17 AND TH17/IFN-Γ CELL NUMBERS INCREASE IN PATIENTS RESPONDING TO INFLIXIMAB THERAPY

Patients with a therapeutic response throughout the eight months of study showed low blood CD4 T cells prior to treatment. CD8 T cells were also low, therefore CD4/CD8 T cell ratio was normal. Absolute numbers of Tfh cells prior to start of infliximab treatment were lower than in controls (p=0.02), and these remained low during therapy. Kinetics of Treg cell numbers were similar between patients and controls, with a parallel increase at week two (Figure 3B, p=0.04) followed by a decline as week 6 and month 8.

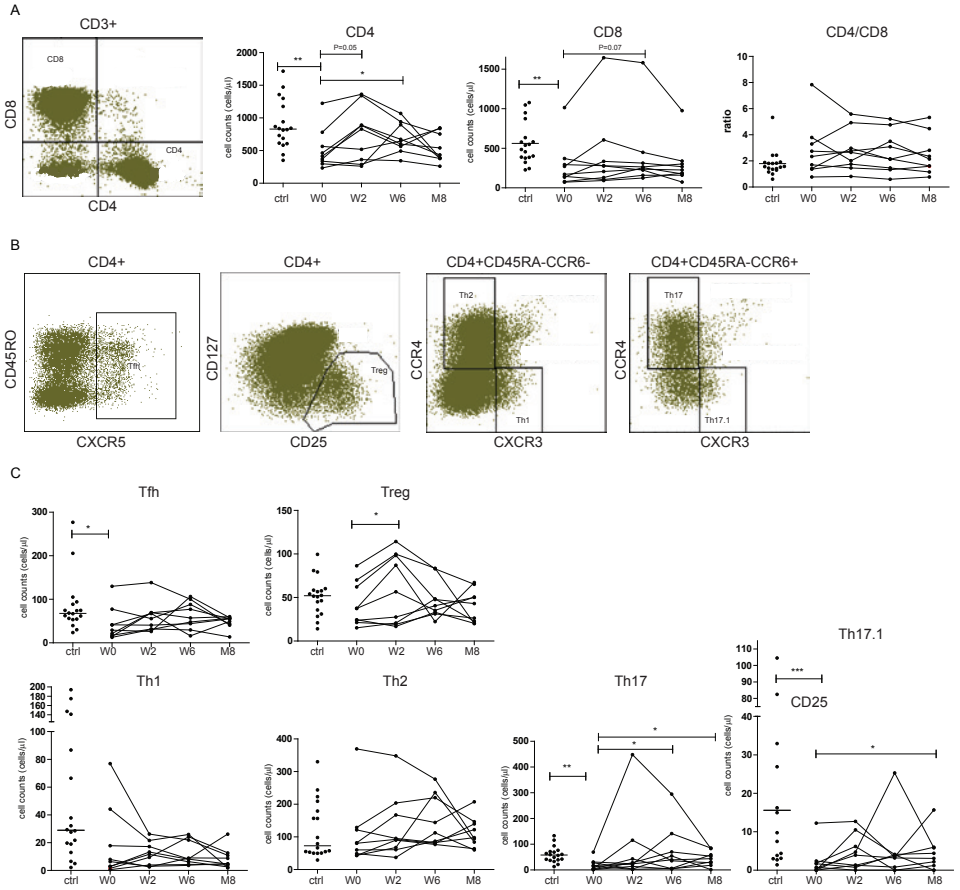


FIGURE 3. T-cell response before and during infliximab therapy in responders to infliximab. **A.** Gating strategy of T-cell subsets including CD4, CD8, CD4/CD8 and absolute counts of CD4, CD8 and CD4/CD8 of healthy controls (ctrl) at baseline (W0) and after 2, 6 weeks (W2, W6) and 8 months (M8) of responders to infliximab therapy (patients 1-8, 10).**B.** Gating of Tfh, Treg, Th1, Th2, Th17 and Th17.1 T cells. **C.** Absolute cell counts of T-cell subsets of healthy controls (ctrl) and patients at baseline (W0) and after 2, 6 weeks (W2, W6) and 8 months (M8) Statistical analysis performed with Mann Whitney U test between controls and baseline patients. Statistical analysis during therapy was performed with a paired analysis with Wilcoxon signed rank test; *, P<0.05; **, P<0.01; ***, P<0.001.

Our cohort did not show differences in Th1 or Th2 cells, whereas both Th17 and Th17.1 (IL-7/IFNγ double producers) cell numbers in patients before therapy were significantly lower than in controls (Figure 3B; p=0.002 and p=0.0004, respectively). In addition, these cell numbers changed during therapy: Th17 cells showed a rapid increase at week 2, lasting for 8 months, whereas Th17.1 cell numbers increased at 8 months.

RAPID NORMALIZATION OF BLOOD IGM MEMORY B CELLS IN PATIENTS RESPONDING TO INFLIXIMAB

As previous studies have demonstrated significant differences in blood memory B cells in patients with sarcoidosis as compared to healthy controls,(19, 24, 31) we here studied how these changes were affected by successful treatment with infliximab. The group of nine remaining patients with a therapeutic response to infliximab had reduced absolute numbers of all six types of memory B cells prior to the start of treatment (Figure 4).

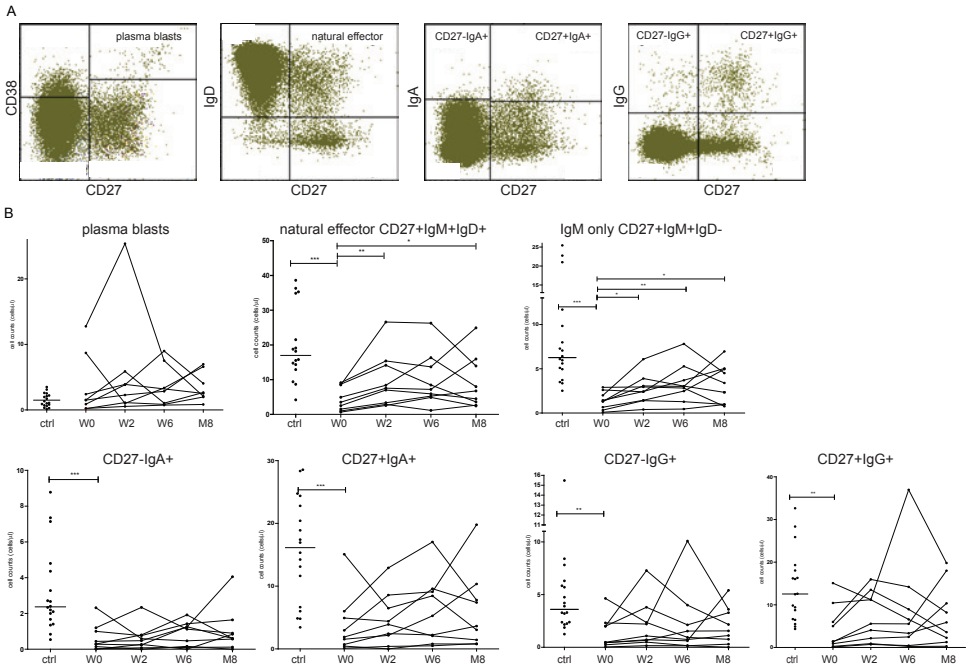


FIGURE 4. B-cell memory before and during therapy in infliximab responders. A. Gating strategy of B-cell subsets within CD19^{+} B cells following exclusion of CD38^{dim}CD21^{low} into: plasma blasts, IgM memory and Ig class-switched memory B cells: CD27^{-}IgA^{+}, CD27^{+}IgA^{+}, CD27^{-}IgG^{+}, CD27^{+}IgG^{+}. **(B).** Absolute cell counts of B-cell subsets of healthy controls (ctrl) and patients at baseline (W0) and after 2, 6 weeks (W2, W6) and 8 months (M8) of responders to infliximab therapy (patients 1-8, 10). Statistical analysis performed with Mann Whitney U test between controls and baseline patients. Statistical analysis during therapy was performed with a paired analysis with Wilcoxon signed rank test; *, P<0.05; **, P<0.01; ***, P<0.001.

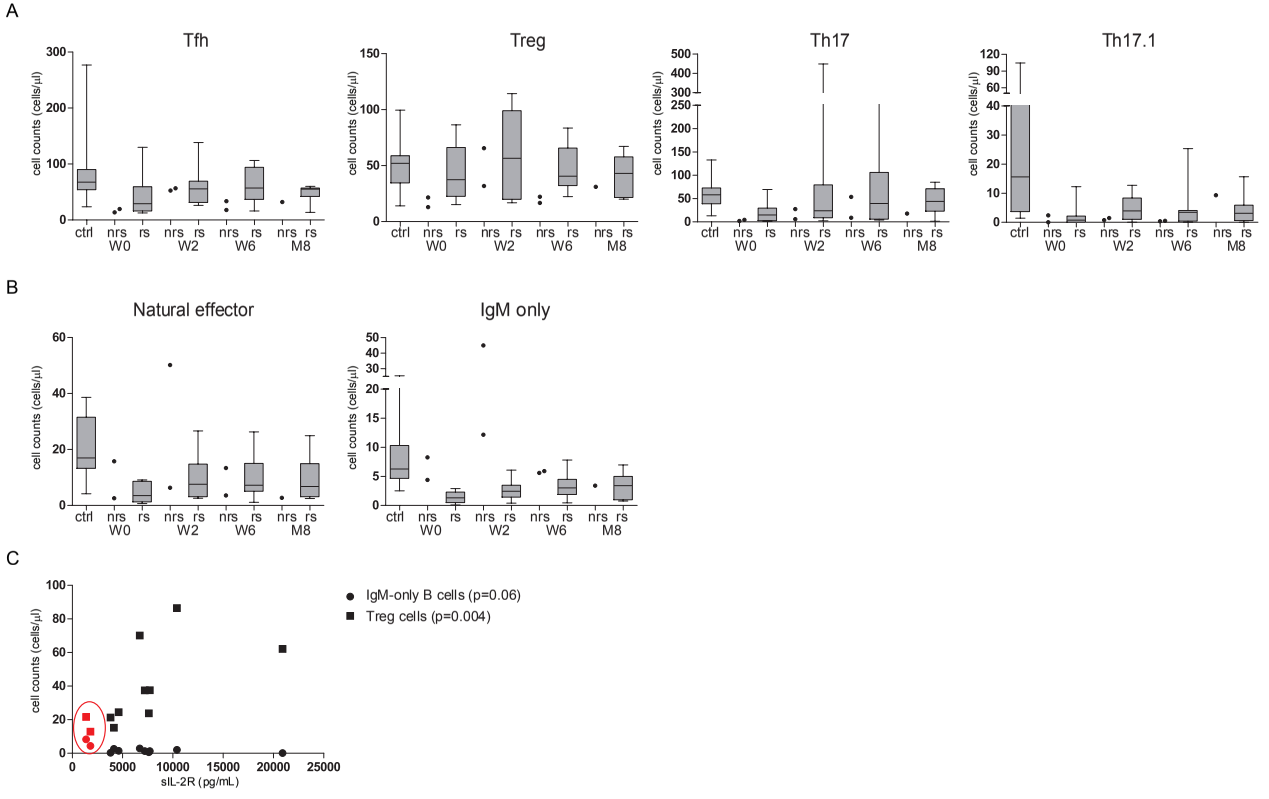


FIGURE 5. T- and B-cell subsets over time in healthy controls, infliximab non-responders and responders. A. absolute numbers of T-cell subsets; Tfh, Treg, Th17 and Th17.1 in healthy controls (ctrl), non-responders (nrs; patient 9 and 11) and responders (rs; patients 1-8, 10) at baseline (W0), 2 and 6 weeks (W2, W6) and 8 months (M8) represented by box and whiskers (min to max). **B.** IgM memory B cells; natural effector and IgM-only B cells in healthy controls (ctrl), non-responders (nrs; patient 9 and 11) and responders (rs; patients 1-8, 10) at baseline (W0), 2 and 6 weeks (W2, W6) and 8 months (M8) represented by box and whiskers (min to max). **C.** Regression analysis of IgM-only B cell (circles) and Treg cell (squares) counts of responders (black), non-responders (red) and sIL-2R levels at baseline using Spearman's rank correlation coefficient.

The numbers of both IgM-expressing memory B cell subsets, ($CD27^+IgM^+IgD^+$ natural effector and $CD27^+IgM^+IgD^-$ IgM-only) memory B cells rapidly increased with a significant rise in week 2 (p=0.004 and p=0.04), that lasted up to month 8 (both p=0.04). For the four Ig class switched memory B-cell subsets ($CD27^-IgA^+$, $CD27^+IgA^+$, $CD27^-IgG^+$ and $CD27^+IgG^+$), no significant change in numbers was detected during treatment. Thus, within the memory-B-cell compartment, specifically the IgM-expressing cells were affected by infliximab therapy.

LOWER LEVELS AT BASELINE OF SIL-2R AND TREG CELLS, AND HIGHER IGM-ONLY B CELLS IN NON-RESPONDERS TO INFLIXIMAB

In the studied period, 2 patients (9 and 11) developed ADA which correlated with clinical response. Those patients were considered non-responders. The most significant related response parameters identified were Tfh, Treg, Th17, Th17.1 and IgM memory B cells. The values of those parameters between responders were compared with those of non-responders and with healthy controls and correlated for response **(**Figure 5A, B**)**. As this group is small, no statistical analysis was performed. Yet, at baseline non-responders showed lower numbers of Tregs and Tfh with higher number of IgM-only B cells in respect to responding patients. When sIL-2R was related to Tregs and IgM-only B cells at baseline; both non responders (in red) showed the lowest sIL-2R levels and Treg numbers while IgM cells were higher **(**Figure 5C**;** p=0.004 and p=0.06, respectively).

NO EFFECT OF INFLIXIMAB THERAPY ON INCREASED SOMATIC HYPERMUTATIONS IN IGA AND IGG TRANSCRIPTS

We previously reported that B cells of patients with sarcoidosis carried increased levels of somatic hypermutations (SHM) in the variable regions of IgA and IgG genes.(24) To study if these levels persist during successful treatment with infliximab, we here analyzed Ig transcripts of three patients before and after ~8 months of treatment with infliximab (Supplemental Figure 1A/C). Similar to the previously reported patients who were not receiving anti-inflammatory medication, our patients carried increased levels of SHM in their IgA transcripts (Figure 6C, p=0.0005) at the start of the study period. This increase remained after 8 months of treatment with infliximab (Figure 6C). A slight, but not significant, increase was seen for SHM levels IgG transcripts of the patients as well (p=0.12). The increase in SHM levels did not result in enhanced selection for replacement mutations in the complement determining region, i.e. the antigen-binding domains (Supplemental Figure 1B,D).

IgG subclass usage changes with age towards increased IgG2 and IgG4 subclasses, and the latter were found to be used more frequently in patients than in controls.(24, 28, 30) In our patients, the combined use of IgG2 and IgG4 before therapy (35%) was

significantly lower than after infliximab treatment (43%; p=0.01). However, for both time points, these were not higher than in controls (47%; Figure 6B). Within IgA, the relative usage of IgA2 in our patients prior to infliximab treatment was similar to controls, but was significantly reduced after 8 months of therapy (Figure 6B, p=0.02).

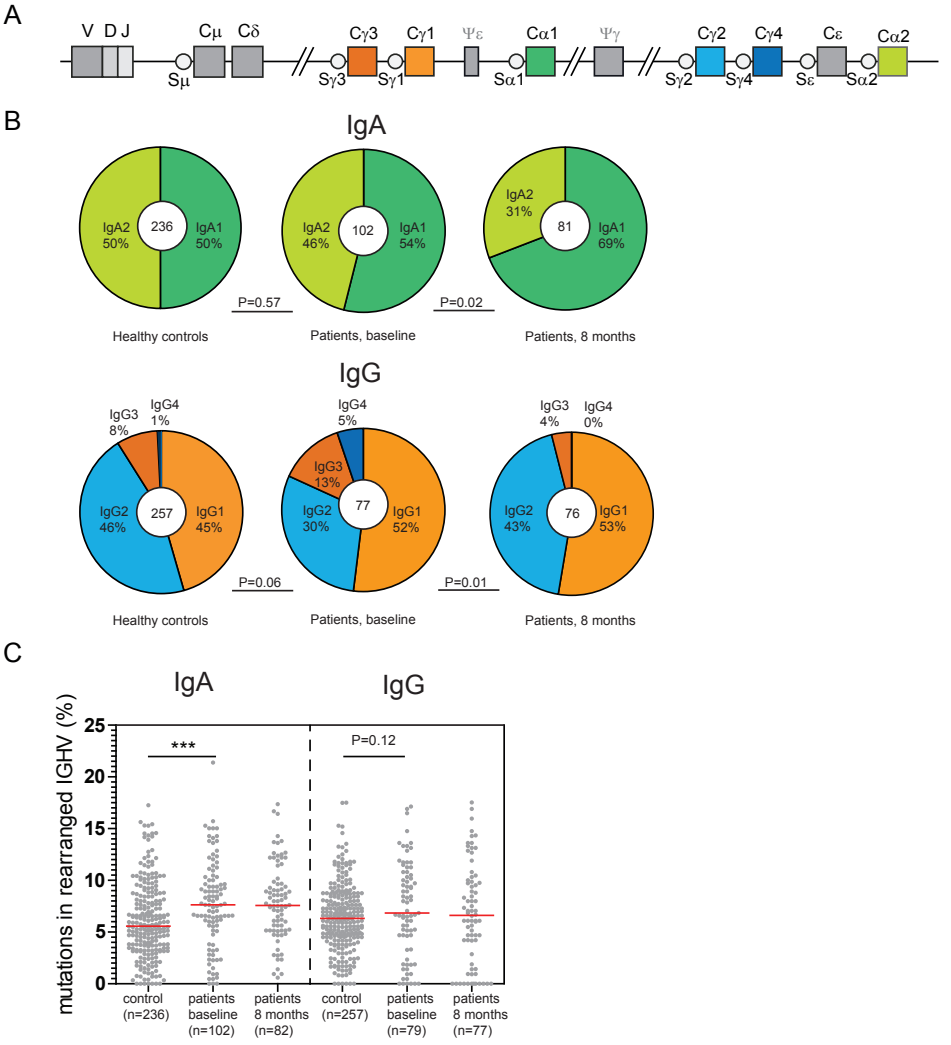


FIGURE 6. Molecular analysis of B cell maturation. A. Schematic representation of the constant region of the *IGH* locus. **B.** Distribution of IgA and IgG subclass usage in switched transcripts of six healthy controls and three patients with sarcoidosis before and during infliximab therapy (patients 1, 2 and 3). Total numbers of analyzed sequences are indicated in the middle of the plots. χ2 Test was performed to analyze differences in distributions. **C.** SHM levels in *IGHV* genes of rearranged IgA and IgG transcripts of healthy controls and patients before and during infliximab therapy at month eight. Grey dots represent unique sequences; red lines represent median values (total numbers of sequences indicated between brackets). Statistical analysis was performed with the Mann-Whitney U test; *, P<0.05; **, P<0.01; ***, P<0.001.

DISCUSSION

Among patients with active sarcoidosis treated with infliximab, infliximab trough levels can be used as therapeutic biomarkers in addition to sIL-2R. sIL-2R levels combined with ADA formation, infliximab trough levels and B- and T-cell kinetics indicate therapeutic success.

TREATMENT OPTIMIZATION – TROUGH LEVELS AND ADA

The therapeutic response in this selective patient cohort consisting of patients with active and severe disease corresponds with that observed in similar a prospective open label studies.(10) Furthermore, most of our patients (7 out of 11) also had extrapulmonary disease, a clinical situation correlated with responses to infliximab.(9-12) Therefore, our cohort would have the potential of more gain in overall clinical scores due to more active inflammation and more extrapulmonary disease prior to treatment.

As sarcoidosis is a rare disease, little has been standardized for infliximab treatment and monitoring.(17) The only two studies examining infliximab trough levels in sarcoidosis have reported a mean of 7.5µg/ml and 18µg/ml between 24 and 26 weeks of infliximab treatment, respectively.(8, 10) In our study, 6 patients had trough levels within the recommended range for other diseases (3-10 µg/ml) 8 months after initiation of therapy. However, levels below 3 µg/ml have not led to therapy failure in two patients on infliximab combined with disease modifying antirheumatic drugs (DMARDs; methotrexate or azathioprine). DMARDs have been shown to prevent ADA formation and loss of response to infliximab in other immune mediated diseases.(32) Interestingly, disease relapse in the two patients in our cohort seemed related to DMARD discontinuation. The 7 patients with a long-lasting therapeutic response all continued their DMARDs, suggesting that co-medication favors a lasting beneficial therapeutic response as is also described in Crohn's disease.(32) The optimization of infliximab therapy through a tailor-made approach measuring ADA and trough levels and using co-medication can enhance the chances of therapeutic response and might therefore be cost-reductive and reduce toxicity as unnecessary high dosage are prevented.(33, 34)

SEROLOGICAL AND CELLULAR MARKERS

Our experiences with sIL-2R as biomarker for therapy monitoring were consistent with those reported earlier.(18, 19) In the presented study, significant decline in sIL-2R levels occurred directly after the first infusion of infliximab. In contrast to sIL2-R, BAFF levels were not significantly elevated as earlier reported in patients with active sarcoidosis.(19, 35) Furthermore, BAFF levels did not change during infliximab therapy either. However,

6/11of our patients had high BAFF levels that were correlated to sIL-2R levels at baseline. IgM memory B-cells, including both the $CD27^+IgM^+IgD^+$ and $CD27^+IgM^+IgD^-$ subsets, increased over time during therapy in patients responding to infliximab. Interestingly, similar results in the pre-switched memory B-cell compartment were found in studies involving rheumatoid arthritis and spondyloarthritis patients upon treatment with TNF-**α** blockers.(36, 37) Salinas et al. proposed that TNF-**α** blockers might influence germinal center reactions leading to decreased isotype switching in spondyloarthritis patients.(37) In this cohort less sequences containing SHM after therapy with infliximab were found.(37) In contrast to this, the presented data in sarcoidosis patients showed stable elevated levels of SHM before and during therapy. The observations of decreased baseline Tfh cells unresponsive to infliximab therapy might indicate that infliximab does not affect germinal center function in sarcoidosis since Tfh cells interact with B cells at these locations.(38) Another potential explanation might be that these memory B cells might accumulate in inflamed sarcoid tissue as has been observed in rheumatoid synovial tissue and therefore are undetectable in peripheral blood.(36) Hypothetically, redistribution from the inflamed tissue to the peripheral blood could also attribute to the increase seen after ignition of TNFα-blockers. Earlier, our group proposed that $CD27^-IgA^+$ cells could be a biomarker for successful infliximab therapy.(24) Actually, in the current study patients already showed low numbers of this cell type at baseline, making it likely that this decrease is more of a general medication effect, rather than a specific infliximab effect.

Increased Tregs in peripheral blood and accumulation in the vicinity of granulomas occur in untreated patients with active sarcoidosis and decrease after effective therapy.(39) Our results in treated patients did not show a difference in absolute Tregs numbers in comparison to controls, however a rapid yet non-lasting increase in Tregs was seen at week 2 after the first infliximab infusion. Another study analyzing the effect of infliximab on Tregs after 14 and 26 weeks, reported also increased percentages of Tregs in sarcoidosis, but with normal absolute numbers without change during therapy.(40) No disturbances were seen in Th1 and Th2 cell numbers, wheras both Th17 and Th17.1 cell numbers were decreased at baseline and increased during therapy. Broos et al. reported decreased frequencies of Th17.1 cells in peripheral blood while these were increased in BAL fluid.(22) The role and function of Th17.1 cells seems to be rather complex, yet our results support the notion that Th17.1 cells more prominently contribute to the pro-inflammatory environment in sarcoidosis than Th1 cells, especially via secretion of IFNγ. Furthermore, increased percentages of Th17.1 cells in BAL fluid in sarcoidosis patients was correlated to developing chronic disease.(41) Our increase of Th17.1 cells during successful infliximab therapy is perhaps a general effect of improvement of disease by clearance of granulomas.

The two patients with a failed response had formation of ADA. Interestingly, these were the only two patients without an elevated baseline sIL-2R plasma level (< 2500

pg/ml), despite having serious and active neurosarcoidosis. Furthermore, compared to infliximab responders, these two patients had lower levels of Tregs at baseline while their IgM-only B cells were within the range of healthy controls. Additionally, the absolute number of Treg and IgM-only B cells were related to sIL-2R baseline levels. Although the group of non-responders is very limited and might be sub-group dependent, our results carefully indicate patients responding well to infliximab have elevated levels of sIL-2R combined with Treg numbers more in line with healthy controls while their IgM memory B cells are substantially reduced.

This study has several strengths, including the thorough immune analysis and individual follow-up both on the short- and long-term. However, the size of our cohort is limited. Yet, especially in this limited group with active and severe disease after failing immunosuppressive medication, the value of infliximab in sarcoidosis is emphasized with most of our patients showing excellent clinical improvement. It would have been valuable if statistical analysis could have been performed with larger subgroups of responders/non-responders. With only 2 patients failing infliximab during this study, statistical analysis was not possible. Furthermore, our group was a varied group of indication for therapy intensification including extra pulmonary involvement. This diversity in disease manifestation is common in daily clinical practice and our results still showed clear patterns irrespective of different disease entities.

In conclusion, infliximab can be a very effective agent in serious and active sarcoidosis. Monitoring the therapeutic effectivity of infliximab combined by sIL-2R, B-and T-cells subsets, ADA and infliximab through levels shows to be promising. Patients responding well to infliximab in general may be characterized by elevated levels of sIL-2R together with Tregs more in line with healthy controls and substantially decreased IgM-only B cells before start of therapy. These findings may implicate that therapeutic monitoring with these biological and pharmacological parameters in patients with active sarcoidosis requiring infliximab could contribute to optimizing infliximab treatment in these patients.

ACKNOWLEDGEMENTS

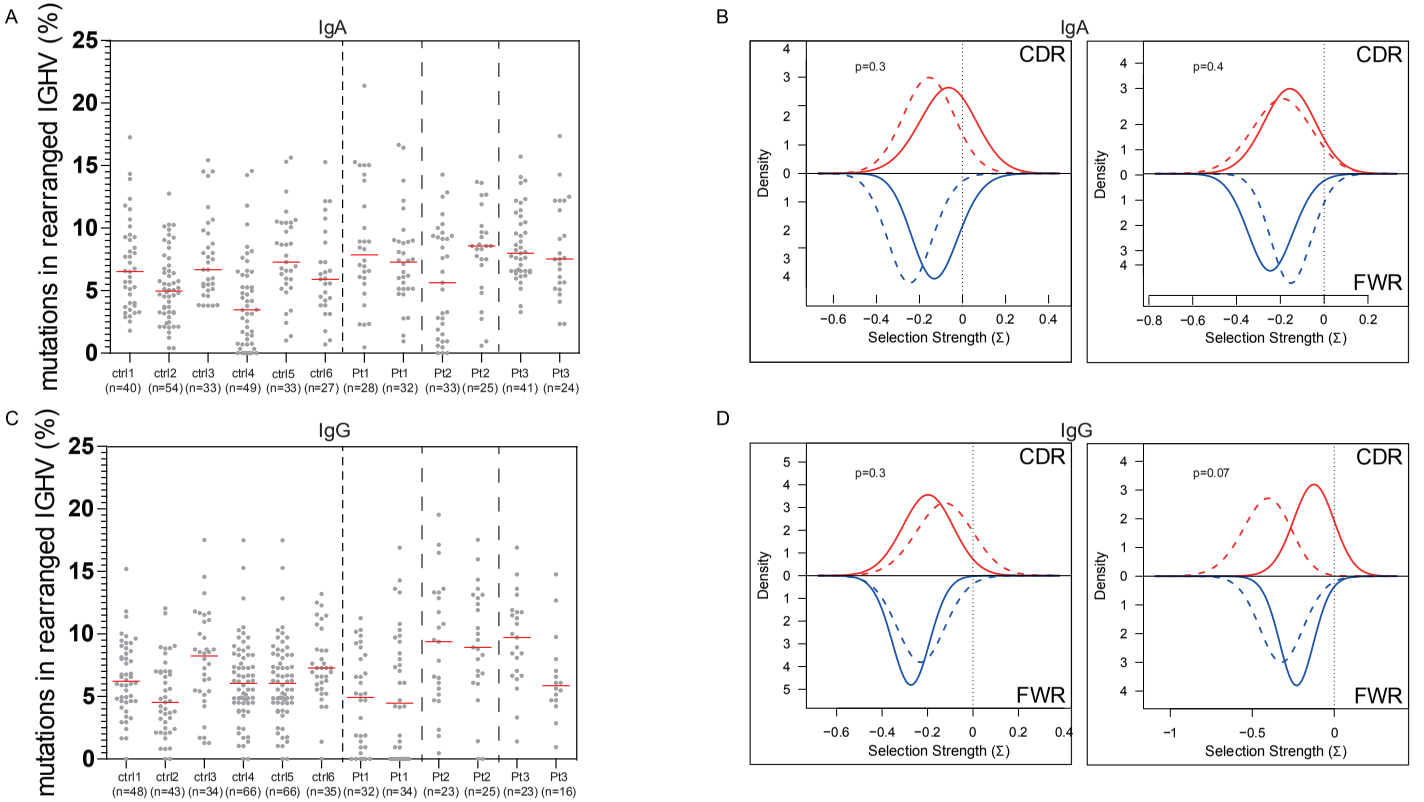
The authors thank Ms. Christina Grosserichter-Wagener and Drs. Jorn J. Heeringa for advice on flowcytometric analysis, Ms. Britt de Jong for her support on SHM analysis and Ms. Nicole Nagtzaam for the sIL-2R measurements. The authors are indebted to the (research)nurses, pulmonologists and clinical immunologists of the Erasmus MC for their help with patient inclusion. Parts of this study were financially supported by the 'Sarcoïdose Belangen Vereniging' (Dutch Sarcoidosis Association). MCvZ is supported by NHMRC Senior Research Fellowship GNT1117687. This study was performed in the framework of the Molecular Medicine Postgraduate School of the Erasmus MC.

REFERENCES

1. Iannuzzi MC, Rybicki BA, Teirstein AS. Sarcoidosis. *N Engl J Med* 2007; 357: 2153-2165.
2. Valeyre D, Prasse A, Nunes H, Uzunhan Y, Brillet PY, Muller-Quernheim J. Sarcoidosis. *Lancet* 2014; 383: 1155-1167.
3. Arango Duque G, Descoteaux A. Macrophage cytokines: involvement in immunity and infectious diseases. *Front Immunol* 2014; 5: 491.
4. Clay H, Volkman HE, Ramakrishnan L. Tumor necrosis factor signaling mediates resistance to mycobacteria by inhibiting bacterial growth and macrophage death. *Immunity* 2008; 29: 283-294.
5. Bean AG, Roach DR, Briscoe H, France MP, Korner H, Sedgwick JD, Britton WJ. Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol Mycobacterium tuberculosis infection, which is not compensated for by lymphotoxin. *Journal of immunology* 1999; 162: 3504-3511.
6. Fehrenbach H, Zissel G, Goldmann T, Tschernig T, Vollmer E, Pabst R, Muller-Quernheim J. Alveolar macrophages are the main source for tumour necrosis factor-alpha in patients with sarcoidosis. *Eur Respir J* 2003; 21: 421-428.
7. Pueringer RJ, Schwartz DA, Dayton CS, Gilbert SR, Hunninghake GW. The relationship between alveolar macrophage TNF, IL-1, and PGE2 release, alveolitis, and disease severity in sarcoidosis. *Chest* 1993; 103: 832-838.
8. Baughman RP, Drent M, Kavuru M, Judson MA, Costabel U, du Bois R, Albera C, Brutsche M, Davis G, Donohue JF, Muller-Quernheim J, Schlenker-Herceg R, Flavin S, Lo KH, Oemar B, Barnathan ES, Sarcoidosis I. Infliximab therapy in patients with chronic sarcoidosis and pulmonary involvement. *Am J Respir Crit Care Med* 2006; 174: 795-802.
9. Judson MA, Baughman RP, Costabel U, Flavin S, Lo KH, Kavuru MS, Drent M, Centocor TSI. Efficacy of infliximab in extrapulmonary sarcoidosis: results from a randomised trial. *Eur Respir J* 2008; 31: 1189-1196.
10. Vorselaars AD, Crommelin HA, Deneer VH, Meek B, Claessen AM, Keijsers RG, van Moorsel CH, Grutters JC. Effectiveness of infliximab in refractory FDG PET-positive sarcoidosis. *Eur Respir J* 2015; 46: 175-185.
11. Chapelon-Abric C, Saadoun D, Biard L, Sene D, Resche-Rigon M, Hervier B, Costedoat-Chalumeau N, Drier A, Leger JM, Cacoub P. Long-term outcome of infliximab in severe chronic and refractory systemic sarcoidosis: a report of 16 cases. *Clin Exp Rheumatol* 2015; 33: 509-515.
12. Russell E, Luk F, Manocha S, Ho T, O'Connor C, Hussain H. Long term follow-up of infliximab efficacy in pulmonary and extra-pulmonary sarcoidosis refractory to conventional therapy. *Semin Arthritis Rheum* 2013; 43: 119-124.
13. D'Haens G, Van Deventer S, Van Hogezand R, Chalmers D, Kothe C, Baert F, Braakman T, Schaible T, Geboes K, Rutgeerts P. Endoscopic and histological healing with infliximab anti-tumor necrosis factor antibodies in Crohn's disease: A European multicenter trial. *Gastroenterology* 1999; 116: 1029-1034.
14. Maini RN, Breedveld FC, Kalden JR, Smolen JS, Davis D, Macfarlane JD, Antoni C, Leeb B, Elliott MJ, Woody JN, Schaible TF, Feldmann M. Therapeutic efficacy of multiple intravenous infusions of anti-tumor necrosis factor alpha monoclonal antibody combined with low-dose weekly methotrexate in rheumatoid arthritis. *Arthritis Rheum* 1998; 41: 1552-1563.
15. Bendtzen K, Geborek P, Svenson M, Larsson L, Kapetanovic MC, Saxne T. Individualized monitoring of drug bioavailability and immunogenicity in rheumatoid arthritis patients treated with the tumor necrosis factor alpha inhibitor infliximab. *Arthritis Rheum* 2006; 54: 3782-3789.

16. Ainsworth MA, Bendtzen K, Brynskov J. Tumor necrosis factor-alpha binding capacity and anti-infliximab antibodies measured by fluid-phase radioimmunoassays as predictors of clinical efficacy of infliximab in Crohn's disease. *Am J Gastroenterol* 2008; 103: 944-948.
17. Zandvliet ML, van Bezooijen JS, Bos MA, Prens EP, van Doorn M, Bijen I, Schreurs MW, van der Velden VH, Koch BC, van Gelder T. Monitoring antigen-specific biologics: current knowledge and future prospects. *Ther Drug Monit* 2013; 35: 588-594.
18. Grutters JC, Fellrath JM, Mulder L, Janssen R, van den Bosch JM, van Velzen-Blad H. Serum soluble interleukin-2 receptor measurement in patients with sarcoidosis: a clinical evaluation. *Chest* 2003; 124: 186-195.
19. Saussine A, Tazi A, Feuillet S, Rybojad M, Juillard C, Bergeron A, Dessirier V, Bouhidel F, Janin A, Bensussan A, Bagot M, Bouaziz JD. Active chronic sarcoidosis is characterized by increased transitional blood B cells, increased IL-10-producing regulatory B cells and high BAFF levels. *PLoS One* 2012; 7: e43588.
20. Hunninghake GW, Crystal RG. Pulmonary sarcoidosis: a disorder mediated by excess helper T-lymphocyte activity at sites of disease activity. *N Engl J Med* 1981; 305: 429-434.
21. Sweiss NJ, Salloum R, Gandhi S, Alegre ML, Sawaqed R, Badaracco M, Pursell K, Pitrak D, Baughman RP, Moller DR, Garcia JG, Niewold TB. Significant CD4, CD8, and CD19 lymphopenia in peripheral blood of sarcoidosis patients correlates with severe disease manifestations. *PLoS One* 2010; 5: e9088.
22. Ramstein J, Broos CE, Simpson LJ, Ansel KM, Sun SA, Ho ME, Woodruff PG, Bhakta NR, Christian L, Nguyen CP, Antalek BJ, Benn BS, Hendriks RW, van den Blink B, Kool M, Koth LL. IFN-gamma-Producing T-Helper 17.1 Cells Are Increased in Sarcoidosis and Are More Prevalent than T-Helper Type 1 Cells. *Am J Respir Crit Care Med* 2016; 193: 1281-1291.
23. Annunziato F, Cosmi L, Santarlasci V, Maggi L, Liotta F, Mazzinghi B, Parente E, Fili L, Ferri S, Frosali F, Giudici F, Romagnani P, Parronchi P, Tonelli F, Maggi E, Romagnani S. Phenotypic and functional features of human Th17 cells. *J Exp Med* 2007; 204: 1849-1861.
24. Kamphuis LS, van Zelm MC, Lam KH, Rimmelzwaan GF, Baarsma GS, Dik WA, Thio HB, van Daele PL, van Velthoven ME, Batstra MR, van Hagen PM, van Laar JA. Perigranuloma localization and abnormal maturation of B cells: emerging key players in sarcoidosis? *Am J Respir Crit Care Med* 2013; 187: 406-416.
25. Berkowska MA, Driessen GJ, Bikos V, Grosserichter-Wagener C, Stamatopoulos K, Cerutti A, He B, Biermann K, Lange JF, van der Burg M, van Dongen JJ, van Zelm MC. Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways. *Blood* 2011; 118: 2150-2158.
26. Heeringa JJ, Karim AF, van Laar JAM, Verdijk RM, Paridaens D, van Hagen PM, van Zelm MC. Expansion of blood IgG4(+) B, TH2, and regulatory T cells in patients with IgG4-related disease. *J Allergy Clin Immunol* 2017.
27. Kalina T, Flores-Montero J, van der Velden VH, Martin-Ayuso M, Bottcher S, Ritgen M, Almeida J, Lhermitte L, Asnafi V, Mendonca A, de Tute R, Cullen M, Sedek L, Vidriales MB, Perez JJ, te Marvelde JG, Mejstrikova E, Hrusak O, Szczepanski T, van Dongen JJ, Orfao A, EuroFlow C. EuroFlow standardization of flow cytometer instrument settings and immunophenotyping protocols. *Leukemia* 2012; 26: 1986-2010.
28. de Jong BG, H IJ, Marques L, van der Burg M, van Dongen JJ, Loos BG, van Zelm MC. Human IgG2- and IgG4-expressing memory B cells display enhanced molecular and phenotypic signs of maturity and accumulate with age. *Immunol Cell Biol* 2017; 95: 744-752.
29. Uduman M, Yaari G, Hershberg U, Stern JA, Shlomchik MJ, Kleinstein SH. Detecting selection in immunoglobulin sequences. *Nucleic Acids Res* 2011; 39: W499-504.
30. Jackson KJ, Wang Y, Collins AM. Human immunoglobulin classes and subclasses show variability in VDJ gene mutation levels. *Immunol Cell Biol* 2014; 92: 729-733.

31. Lee NS, Barber L, Akula SM, Sigounas G, Kataria YP, Arce S. Disturbed homeostasis and multiple signaling defects in the peripheral blood B-cell compartment of patients with severe chronic sarcoidosis. *Clin Vaccine Immunol* 2011; 18: 1306-1316.
32. Vermeire S, Noman M, Van Assche G, Baert F, D'Haens G, Rutgeerts P. Effectiveness of concomitant immunosuppressive therapy in suppressing the formation of antibodies to infliximab in Crohn's disease. *Gut* 2007; 56: 1226-1231.
33. Pavelka K, Jarosova K, Suchy D, Senolt L, Chroust K, Dusek L, Vencovsky J. Increasing the infliximab dose in rheumatoid arthritis patients: a randomised, double blind study failed to confirm its efficacy. *Ann Rheum Dis* 2009; 68: 1285-1289.
34. Zavada J, Uher M, Sisol K, Forejtova S, Jarosova K, Mann H, Vencovsky J, Pavelka K. A tailored approach to reduce dose of anti-TNF drugs may be equally effective, but substantially less costly than standard dosing in patients with ankylosing spondylitis over 1 year: a propensity score-matched cohort study. *Ann Rheum Dis* 2016; 75: 96-102.
35. Ueda-Hayakawa I, Tanimura H, Osawa M, Iwasaka H, Ohe S, Yamazaki F, Mizuno K, Okamoto H. Elevated serum BAFF levels in patients with sarcoidosis: association with disease activity. *Rheumatology (Oxford)* 2013; 52: 1658-1666.
36. Souto-Carneiro MM, Mahadevan V, Takada K, Fritsch-Stork R, Nanki T, Brown M, Fleisher TA, Wilson M, Goldbach-Mansky R, Lipsky PE. Alterations in peripheral blood memory B cells in patients with active rheumatoid arthritis are dependent on the action of tumour necrosis factor. *Arthritis Res Ther* 2009; 11: R84.
37. Salinas GF, De Rycke L, Barendregt B, Paramarta JE, Hreggvidstdottir H, Cantaert T, van der Burg M, Tak PP, Baeten D. Anti-TNF treatment blocks the induction of T cell-dependent humoral responses. *Ann Rheum Dis* 2013; 72: 1037-1043.
38. Breitfeld D, Ohl L, Kremmer E, Ellwart J, Sallusto F, Lipp M, Forster R. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J Exp Med* 2000; 192: 1545-1552.
39. Miyara M, Amoura Z, Parizot C, Badoual C, Dorgham K, Trad S, Kambouchner M, Valeyre D, Chapelon-Abric C, Debre P, Piette JC, Gorochov G. The immune paradox of sarcoidosis and regulatory T cells. *J Exp Med* 2006; 203: 359-370.
40. Verwoerd A, Hijdra D, Vorselaars AD, Crommelin HA, van Moorsel CH, Grutters JC, Claessen AM. Infliximab therapy balances regulatory T cells, tumour necrosis factor receptor 2 (TNFR2) expression and soluble TNFR2 in sarcoidosis. *Clin Exp Immunol* 2016; 185: 263-270.
41. Broos CE, Koth LL, van Nimwegen M, In 't Veen J, Paulissen SMJ, van Hamburg JP, Annema JT, Heller-Baan R, Kleinjan A, Hoogsteden HC, Wijsenbeek MS, Hendriks RW, van den Blink B, Kool M. Increased T-helper 17.1 cells in sarcoidosis mediastinal lymph nodes. *Eur Respir J* 2018; 51.
42. Yaari G, Uduman M, Kleinstein SH. Quantifying selection in high-throughput Immunoglobulin sequencing data sets. *Nucleic Acids Res* 2012; 40: e134.



SUPPLEMENTAL FIGURE 1. SHM analysis in IGHV regions of IgA and IgG transcripts. SMH levels in IGHV genes of rearranged IgA **(A)** and IgG **(C)** transcripts of six healthy controls (ctrl) and three patients (patients 1, 2 and 3). Grey dots represent unique sequences; red lines represent median values. Selection for replacement mutation for IgA sequences **(B)** and IgG sequences **(D)** in IGHV-CDR (red line) and IGHV-FR regions (blue line) as determined with the BASELINe program.(29, 42) Solid lines represent patients, dashed lines represent healthy controls.


Flinders Street
9

CHAPTER 5

EFFECTIVENESS AND SAFETY OF INFLIXIMAB IN PATHOLOGY CONFIRMED NEUROSARCOIDOSIS

D. Fritz[1]*, W.M.C. Timmermans[2,3]*, J.A.M. van Laar[2,3], P. M. van Hagen[2,3], D. Siepman[4], D. van de Beek[1], M.C. Brouwer[1]

[1] Academic Medical Center, Department of Neurology, Amsterdam, The Netherlands;
[2] Erasmus Medical Center, Department of Internal Medicine, Rotterdam, The Netherlands
[3] Erasmus Medical Center, Department of Immunology, Rotterdam, The Netherlands
[4] Erasmus Medical Center, Department of Neurology, Rotterdam, The Netherlands

**authors contributed equally*

Manuscript in preparation

ABSTRACT

Introduction

Neurosarcoidosis is a manifestation of sarcoidosis with inflammatory involvement of the nervous system. Infliximab is a tumor necrosis factor-alpha (TNF) blocker and used as a third line treatment for sarcoidosis.

Methods

In a retrospective study in two tertiary referral centres in the Netherlands, data were collected data on clinical characteristics and outcome of patients with biopsy proven neurosarcoidosis who were treated with infliximab.

Results

A total of 28 patients were identified with a mean age at baseline of 42 of whom 16 (57%) were male. The clinical presentation of severe neurosarcoidosis at the start of infliximab treatment was consistent with cerebral parenchyma localization (16/28 patients, 59%), pituitary gland/hypothalamic sarcoidosis (15/28, 54%), peripheral nerves involvement (12/28, 43%) and chronic meningitis (11/28, 41%). Infliximab treatment led to complete remission in 6 patients (21%), improvement in 14 (50%), stable disease in 7 (25%) and deterioration in 1 (4%). At the end of follow-up with a median of 32 months, 5 patients (18%) had died. Successful tapering or discontinuation of corticosteroids was achieved in 19 of 28 patients (68%). In patients of whom the dosage of infliximab was decreased or discontinued, a relapse occurred in 5 of 19 patients (26%). Complications of infliximab were reported in 10 of 28 patients (36%) and consisted of infections in 8 (29%) and elevated liver tests and an allergic reaction both in 1 (4%).

Conclusion

Infliximab is an effective and relatively safe third line treatment option in neurosarcoidosis and leads to remission or improvement in the majority of patients. However, relapses may occur in patients in whom infliximab was either tapered or discontinued.

INTRODUCTION

Sarcoidosis is a multisystem disorder and is characterized by the presence of granulomas that can affect every organ system.[1] The prevalence of sarcoidosis is estimated to be between 5-50 per 100.000 of the population with the highest prevalence in Northern Europe.[1] Approximately 5% of sarcoidosis patients have neurosarcoidosis in which granulomas involve the nervous system. [2] Neurosarcoidosis is a severe form of sarcoidosis in which one-third of patients either remain stable, deteriorate or die despite immunosuppressive treatment.[2] No clinical trials have been performed in neurosarcoidosis patients and treatment choices are mainly based on evidence from non-neurological sarcoidosis.

In patients with neurosarcoidosis refractory to first or second-line treatment with infliximab, a tumor necrosis factor alpha (TNF) blocker has emerged as a treatment option in the past years.[3] TNF-α is a pivotal pro-inflammatory cytokine produced by macrophages and activated T-cells and plays a central role in the formation and maintenance of granulomas.[4] Corticosteroids lead to decreased TNF-α excretion by alveolar macrophages which may indicate a therapeutic effect in patients with pulmonary sarcoidosis.[5, 6] This rationale led to two randomized controlled clinical trials evaluating the efficacy of infliximab in chronic pulmonary sarcoidosis.[7, 8] These trials showed mixed results, with a statistically significant improvement of the predicted forced vital capacity at 24 weeks after initiation in one study, albeit small with 2.5%.[7] However, chronic steroid-responsive sarcoidosis patients with nervous system involvement may profit from infliximab treatment.[9] Moreover, a beneficial effect has consequently been described in several case reports and two retrospective cohort studies.[3, 10, 11] To substantiate this potential beneficial effect of TNF-blockers we analyzed the use of infliximab in biopsy proven neurosarcoidosis patients and evaluate the treatment response and safety in a large multicenter tertiary center cohort.

METHODS

A retrospective study was performed with inclusion of all patients with biopsy proven sarcoidosis and neurological involvement who were treated with infliximab before the 1st of June 2017 at the Academic Medical Center (AMC) in Amsterdam and the Erasmus Medical Center (EMC) in Rotterdam, two tertiary referral centers for (neuro)sarcoidosis in the Netherlands. Ethical approval is not required in the Netherlands for a retrospective study with anonymized patient data such as our study. Patients were identified by their treating physician and data was collected in a database. The diagnosis of neurosarcoidosis was based on the *Zajicek* criteria, later modified by *Tavee*.[12, 13] In this study we included only cases with biopsy proven neurosarcoidosis. A positive histology for sarcoidosis was

defined as the presence of histological features consistent with sarcoidosis defined as non-caseating granulomas with epithelioid cells and macrophages.[6]

For all patients, a case record form was created containing baseline characteristics, disease course and immune modulating medication used at baseline, clinical characteristics and results of ancillary investigations at baseline, infliximab treatment and treatment response, disease course and clinical outcome up to the last time of follow-up and adverse events. Baseline was defined as the initiation of infliximab treatment. All patients were treated with a dosage of 5mg/kg. In the EMC patients were treated at week 0, 2, 6 during the induction phase, followed by an infusion once every 4 to 8 weeks based on the clinical features and seriousness by their treating physician. In the AMC patients did not undergo an induction phase and immediately received infusions once every 4 to 8 weeks based on the clinical features and seriousness. All patients were initially treated with Remicade® and were switched in 2016 to the biosimilar Remsima®. All infections, infusion reactions and laboratory abnormalities that occurred during the use of infliximab were reported. The response rate to treatment in each case was scored as "improvement on therapy", "stable disease" (*e.g.*, unchanged compared to clinical situation prior to treatment), "deterioration" and "spontaneous improvement" (*e.g.*, improvement in patients not treated for neurosarcoidosis or improvement after ceasing treatment in patients). Clinical outcome was graded into functional disability at the last recorded presentation in in- or outpatient setting. The functional disability in each case was scored, using the Modified Rankin Scale (mRS), as "asymptomatic", "complaints without functional disability", "complaints with minor functional disability" (e.g. neurological deficits mildly interfering in everyday life, such as inability to cycle due to motor dysfunction), "complaints with moderate-to-severe functional disability" (e.g. neurological deficits interfering everyday life, resulting in failure to return to job or school, requirement of special equipment such as crutches or a wheelchair, or assistance with everyday activities) and death.

Statistical analysis was performed to compare differences between groups using the Fisher's exact test for dichotomous variables and binary logistic regression for ordinal and continuous variables. A p-value <0.05 was considered significant.

RESULTS

CLINICAL CHARACTERISTICS

A total of 28 patients were included, 11 in the AMC and 17 in the EMC. Baseline characteristics, clinical manifestations and ancillary investigations at the start of infliximab are described in table 1 and were similar in both centers. The median time of follow-up was 32 months (IQR 17-54). The included patients had a mean age at baseline of 42 (SD 10.3)

and 16 (57%) were male. Of these patients 16 (57%) were Caucasian, 6 (21%) of African descent and 6 (21%) had other ethnic backgrounds. Neurological involvement at the start of infliximab consisted of parenchymal involvement in 16 patients (59%), pituitary/hypothalamic involvement in 15 (54%), peripheral nerve involvement in 12 (43%), chronic meningitis in 11 (41%), cranial nerve palsy in 7 (25%), hydrocephalus in 6 (22%), spinal cord involvement in 5 (18%) and muscle involvement in 1 patient (4%). The majority of patients had systemic involvement including lymph node (27%), intrapulmonary (27%) and ophthalmologic (22%) involvement. According to the Zajicek criteria 2 patients were diagnosed with definite and 26 patients with probable neurosarcoidosis.

TABLE 1. Baseline characteristics and disease course at baseline

Characteristic	n/N (%)	Characteristic	n/N (%)
Age at baseline (SD), years	42 (10.3)	Ancillary investigations	
Male sex	16 (57)	Serum ACE elevated	0/12 (0)
Ethnicity		Serum sIL2r elevated	4/6 (67)
Caucasian	16 (57)	Serum CRP elevated	7/21 (33)
African descent	6 (21)	Serum ESR elevated	8/19 (42)
Other[a]	6 (21)	CSF leukocytes elevated	6/6 (100)
Neurological involvement		CSF protein elevated	6/6 (100)
Parenchymal	16/27 (59)	Chest CT suggestive	3/4 (75)
Neuro-endocrine	15/27 (56)	^{18}F-FDG PET-CT suggestive	1/6 (16)
Peripheral nerve	12/27 (44)	MRI brain suggestive	21/22 (95)
Chronic aseptic meningitis	11/27 (41)	MRI spinal cord suggestive	2/7 (29)
Cranial nerve palsy	7/27 (26)	Immunosuppressant use at baseline	
Hydrocephalus	6/27 (22)	Corticosteroids	11/28 (39)
Spinal cord	5/27 (19)	Corticosteroids + methotrexate	6/28 (21)
Muscle	1/27 (4)	Corticosteroids + azathioprine	4/28 (14)
Systemic involvement[e]		Methotrexate	2/28 (7)
Lymph node	18/25 (72)	Corticosteroids + hydrochloroquine	1/28 (4)
Lungs	7/26 (27)	Corticosteroids + MMF	2/28 (7)
Eye	6/27 (22)	None	3/28 (11)
ENT	3/28 (11)	Total immunosuppressant use before IFX	
Skin	2/27 (7)	Corticosteroids	28/28 (100)
Joints	1/28 (4)	Methotrexate	14/28 (50)
Zajicek criteria		Azathioprine	13/28 (46)
Definite neurosarcoidosis	2/28 (7)	Mycophenolate Mofetil	5/28 (18)
Probable neurosarcoidosis	26/28 (93)	Cyclophosphamide	2/28 (7)
		Hydroxychloroquine	1/28 (4)
		Cyclosporine	1/28 (4)

[a] Other ethnicities: 2 (7%) North African 2 (7%) Hindustan, 1 (4%) Asian and 1 (4%) Hispanic

Treatment used at the start of infliximab consisted of corticosteroids in 11 patients (39%), prednisolone and methotrexate in 6 (21%), prednisolone and azathioprine in 4 (14%), methotrexate and prednisone with mycophenolate mofetil in 2 (7%) and prednisone with hydrochloroquine in 1 patient (4%). Three patients were not receiving immunosuppressive medication at the start of infliximab, but had received this previously. Before the start of infliximab, all patients had been treated with corticosteroids, 14 (50%) with methotrexate, 13 (46%) with azathioprine and 5 (18%) with mycophenolate mofetil, 2 (7%) with cyclophosphamide and 1 (4%) with hydroxychloroquine or cyclosporine. Overall, 24 of 28 patients (86%) had been treated with second line treatment previously (table 2).

TABLE 2. Treatment and outcome

Characteristic	n/N (%)	Characteristic	n/N (%)
Duration of infliximab treatment in months	26 (18)	1st or 2nd line treatment change	
Total number of infliximab infusions	XX (8-30)	Taper of 1st line treatment	6/28 (21)
Infliximab dosage		Stop of 1st line treatment	13/28 (46)
5mg/kg 1x/4 weeks	5/28 (18)	Stop of 2nd line treatment	4/28 (14)
5mg/kg 1x/6 weeks	12/28 (43)	Auto-antibodies	1/5 (20)
5mg/kg 1x/8 weeks	11/28 (39)	IFX dosage decrease or stop	19/28 (68)
Reason to start with infliximab		Good treatment response	8/28 (29)
Relapse when tapering 1st line, despite 2nd line	16/28 (57)	Insufficient treatment response	2/28 (7)
Serious side effects 1st or 2nd line treatment	8/28 (29)	Major side effects	3/28 (11)
Chronic progression despite 1st or 2nd line	3/28 (11)	Stable symptoms, no disease activity	4/28 (14)
Relapse after tapering 1st line	1/28 (4)	Other	2/28 (7)
Treatment response		No change/stop IFX	9/28 (32)
Remission	6/28 (21)	Result of change/stop IFX	
Improvement	14/28 (50)	None	11/19 (58)
Stable disease	7/28 (25)	Relapse	5/19 (26)
Deterioration	1/28 (4)	Unknown	3/19 (16)
Change of sIL2r		Complications of IFX treatment	10/28 (36)
Improved to normal levels	9/14 (64)	Infections	8/28 (29)
Remained elevated	2/14 (14)	Elevated liver tests	1/28 (4)
Remained stable	2/14 (14)	Allergic reaction	1/28 (4)
Deteriorated to abnormal	1/14 (7)	Modified ranking score	
Change of neurological imaging		No disability	7/28 (25)
Improvement	15/21 (71)	Low disability	9/28 (32)
Stable	4/21 (19)	Moderate disability	6/28 (21)
Other abnormalities	2/21 (10)	Severe disability	1/28 (4)
		Death	5/28 (18)

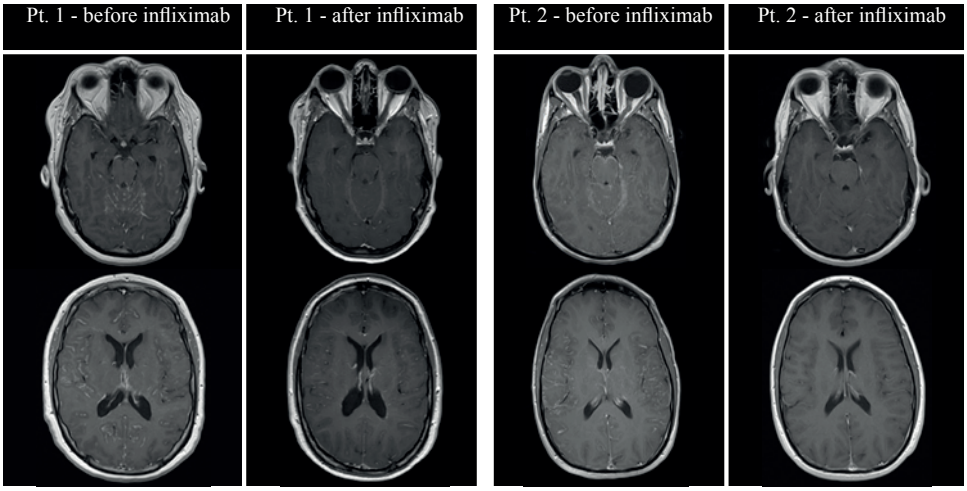


FIGURE 1. Results of MRI brain before and after start of infliximab Contrast enhanced MRI T1 sequence before and during infliximab of two patients of the AMC cohort (patient 10 and 11). Both patients had a complete clinical and radiological response to infliximab.

THERAPEUTIC OUTCOME

The starting dose of infliximab was 5 milligrams per kilogram in all patients and was given once every 4 weeks in 5 patients (18%), once every 6 weeks in 12 patients (43%) and once every 8 weeks in 11 patients (39%). The median total number of infusions was 17 (interquartile range [IQR] 8 – 39) and infliximab treatment had a median duration of 23 months (IQR 12 – 38). The most frequent reason to start infliximab was a relapse when tapering corticosteroids despite second line treatment in 16 of 28 patients (57%). Other reasons were serious side effects of first and/or second line treatment in 8 (29%), chronic progression despite first and/or second line treatment in 3 patients (11%) and a relapse after tapering corticosteroids in 1 patient (4%). The treatment response consisted of remission in 6 patients (21%), improvement in 14 patients (50%), stable disease in 7 patients (25%) and death in 1 patient (4%). Treatment responses were similar in both centers. The sIL-2R values returned to normal levels in 9 (64%) and improved in 2 of 14 patients (14%). Neurological MRI was repeated in 21 patients at follow-up and radiological abnormalities attributed to neurosarcoidosis decreased in 15 (71%), remained stable in 4 patients (19%) and deteriorated in one patient (7%). Additionally, one patient developed hydrocephalus. A favorable treatment response was not dependent of ethnicity (p=0.85), age at the start of infliximab (p=0.13) and time between diagnosis neurosarcoidosis and the start of infliximab (p=0.08) in a logistic regression analysis. Furthermore, a favorable treatment response was not significantly associated with the use of second line therapy in combination with infliximab (13/15 [87%] versus 7/13 [54%], p=0.10). A favorable treatment response was seen more

frequently in men compared to women (14/16 [88%] versus (6/12 [50%], p=0.044). First line therapy was tapered in 6 of 28 patients (21%) and stopped in 13 patients (46%), without signs of disease reoccurrence. Second line treatment was stopped in 4 (14%) and did not change in 9 of 28 patients (32%). The infliximab dosage was decreased in 4 of 27 patients (15%) during follow up and was stopped in 15 of 27 patients (56%).

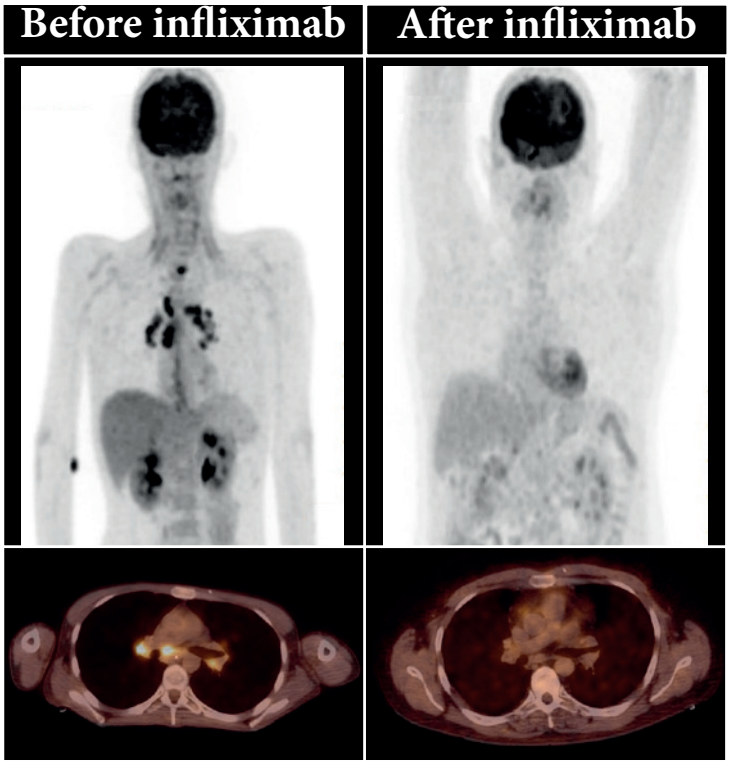


FIGURE 2. Systemic imaging before and after start of infliximab 18F-FDG PET/CT imaging of patient 2 of the AMC cohort, before and after the start of infliximab showing a decreased uptake of hilar FDG avidity and decrease in lymphadenopathy.

This was done due to a good treatment response without signs of disease activity in 8 patients (29%), stable disease without signs of disease activity in 4 patients (14%), major side effects in 3 patients (11%) and insufficient treatment response in 2 patients (7%). In patients of whom the dosage was decreased or the infliximab was discontinued, a relapse was reported in 5 of 19 patients (26%), which was not associated with the length of infliximab use before discontinuation. Infliximab was restarted or dosage was increased in all these patients leading to a favorable treatment in four patients and stable disease in 1 patient. Of the remaining 9 patients in whom infliximab was continued a relapse occurred in 1 of 9 patients (11%), which was attributed to the stop of methotrexate because of liver toxicity. Auto-antibodies against infliximab were

tested in 5 patients who experienced a relapse and was positive in three patients. These patients were switched to adalimumab, which led to improvement in two patients and stable disease in one patient. At the last time of follow up, 7 of 28 patients (25%) had no disability, 9 (32%) had low disability, 6 (21%) had moderate disability, 1 patient (4%) had severe disability and 5 (18%) died. Of the patients who died, cause of death was sepsis in two patients and brain stem hemorrhage, malignancy and an unknown cause of death in one patient. Of these patients, only the two patients with infectious complications used infliximab at the time of death and both were on concomitant corticosteroid therapy. Complications attributed to infliximab treatment occurred in 10 of 28 patients (36%) and consisted of infections in 8 patients (29%), elevated liver tests and an allergic reaction both in 1 patient (4%). Infections consisted of pneumonia in 4 patients, urinary tract infection in combination with pneumonia in 1 patient and urinary tract infection in 1 patient, all necessitating hospital admission. Three out of 6 patients with infections also used corticosteroids and 2 already suffered from these infections before the start of infliximab. Three patients with infections eventually died during follow-up. In 3 of the 15 patients (20%) in whom infliximab was stopped this was due to side effects, consisting of elevated liver tests, recurrent infections or an allergic reaction.

DISCUSSION

In this multicenter retrospective cohort study, we report a favorable long-term outcome and safety of neurosarcoidosis patients treated with infliximab. To date, the evidence for the use of infliximab in neurosarcoidosis consists of case reports and two retrospective multicenter cohort studies describing 18 and 66 patients with probable or definite neurosarcoidosis.[10, 11] Both papers described treatment responses with improvement or remission in 89% and 77% of patients respectively, which is comparable to those described in the present study.[10,11] Naam *et al.* described an inversed correlation between the duration of neurosarcoidosis and a favorable treatment response. This finding could not be reproduced in this cohort, however given the severity of the neurologic manifestation it seems reasonable to start infliximab when first line treatment fails and a quick treatment response is required or when second line treatment fails.

In our cohort starting infliximab was followed by tapering of concomitant first line treatment in 67% of patients and eventually complete discontinuation of corticosteroids in 46% of patients. This is in line with another study describing discontinuation of steroids after the start of infliximab in 40% of the patients and maintenance of prednisone on 5 milligrams per day or less in 27% of the patients.[11] This suggests that infliximab has prednisone sparing effects, which is in accordance to trials performed in patients with refractory or severe Crohn's disease in which infliximab is found to increase the steroid free interval.[14] Furthermore, this is important given the side effects associated with

long-term corticosteroids use.[15]

Currently, no international guidelines exist on how to proceed after a favorable treatment response following infliximab treatment. In patients in whom infliximab treatment was stopped or dosage was decreased, one-fourth had a relapse of disease activity. This is a lower relapse rate then described previously in 56% of the patients.[11] Observational studies describing the use of infliximab in patients with a clinical remission of Crohn's disease, found relapse rates of between 40 and 50% of patients after infliximab cessation.[16] These data stresses the importance of monitoring disease activity after infliximab cessation, although it remains unknown if there are predictors for relapse in neurosarcoidosis. In case of a relapse, dosage can be increased and/or intervals between infusions can be shortened quickly. In our cohort, when patients were reintroduced to infliximab, they were likely to again show a good treatment response, similar as seen in inflammatory bowel disease.[17] Also, it is essential to assess auto-antibody formation and infliximab trough levels when a patient shows signs of a relapse.[18, 19] Concomitant methotrexate is advised as this has been shown to be efficient in reducing immunogenicity.[20] Furthermore, combination therapy with another steroid-sparing agent may be associated with a favorable treatment response to infliximab.[11]

An important side effect of infliximab is the occurrence of infections in 29% of the patients, which is a well-known complication. Importantly, 2 patients died of sepsis when using infliximab during follow-up, however it is not known whether it is only the effect of infliximab or merely the concomitant use of corticosteroids in these patients. The infection rate is in accordance to other studies describing infliximab use in neurosarcoidosis varying between 10 and 39%, however in these studies none of the patients died due to infectious complications.[10, 11] In a phase 2 randomized controlled trial assessing the efficacy of infliximab in non-neurological sarcoidosis, authors described infections in 54 of 91 patients (59%) of which 11 were defined as serious.[7] In a larger randomized controlled trial performed in patients with moderate to severe Crohn disease, infections were reported in 44% of patients treated with infliximab of which 10% were defined as serious.[14] The risk of infectious complications is considered to be higher when patients are treated in combination with corticosteroids or other immunosuppressive drugs.[21, 22] This data suggest that tapering corticosteroids remains essential when signs of disease activity have diminished. Sequentially, infliximab infusions can be delayed and stopped. One study found that most opportunistic infections occur in the first year of treatment, stressing the importance of preventing screening of opportunistic infections before the start of infliximab, most importantly tuberculosis as is routinely performed.[21]

In our cohort both hospitals treated patients with the biosimilar Remsima from 2016 onwards for both new and patients already receiving infliximab. To date, there is limited evidence on the use of biosimilars in sarcoidosis. An expert opinion paper advised that patients who were not treated before with infliximab may safely start with

a biosimilar, however the authours advised to avoid switching patients to biosimilars when already using infliximab.[23] Furthermore, in a small retrospective cohort study biosimilar Inflectra showed comparable efficacy and safety compared to Remicade in patients with refractory sarcoidosis.[24] Although our cohort is small, our study does not provide evidence for the advice not to switch patients already using infliximab, as was to be expected from large studies in other immune mediated diseases.[25, 26]

Our study has several limitations. Firstly, both the retrospective and multicenter approach of our study resulted in heterogeneous assessment of disease activity, treatment strategies and outcome, as well as missing data in some patients. Furthermore, treatment strategies differed between the two centers. In the EMC an induction phase used when they started patients on infliximab, while the AMC initiates treatment once every 4 to 8 weeks without an initial induction phase. Despite these differences, baseline characteristics and treatment response did not differ between the two centers. However, sIL-2R measurements were only performed in the EMC cohort. These limitations are inherent to the study design and to overcome the problem of missing data we gave an overall of n of N (%) in presenting our results. In addition, a majority of patients were treated with first – and/or second line therapy possibly contributing to treatment responses and the occurrence of side effects. Lastly, we included only patients treated in our tertiary referral centers, introducing a selection bias. We feel that neurosarcoidosis patients treated with infliximab must be treated at specialized centers, which is the norm in the Netherlands.

In conclusion, these results provide additional evidence for the use of infliximab in neurosarcoidosis as our results suggest that it is effective and can prevent long term use of high dose corticosteroids reducing long-term side effects. Physicians should be aware of possible side effects as seen in 36% of patients in our cohort, with a risk of serious infectious complications. In addition to the high response rate to infliximab, this study provides further evidence that infliximab is relatively safe to use in neurosarcoidosis. Most importantly, it remains essential to perform prospective cohort studies and randomized controlled trials in neurosarcoidosis patients. These should shed light over when to start with infliximab, whether mono- or combination therapy is preferred and when to decrease and/or stop infliximab dosage during follow-up. Given the high relapse rate after discontinuation of infliximab, possible predictors of relapses are warranted.

REFERENCES

1. Valeyre D, Prasse A, Nunes H, Uzunhan Y, Brillet PY, Muller-Quernheim J. Sarcoidosis. Lancet 2014;383:1155-1167.
2. Fritz D, van de Beek D, Brouwer MC. Clinical features, treatment and outcome in neurosarcoidosis: systematic review and meta-analysis. BMC Neurol 2016;16:220.
3. Fritz D, Voortman M, van de Beek D, Drent M, Brouwer MC. Many faces of neurosarcoidosis: from chronic meningitis to myelopathy. Curr Opin Pulm Med 2017;23:439-446.
4. Baughman RP, Iannuzzi M. Tumour necrosis factor in sarcoidosis and its potential for targeted therapy. BioDrugs 2003;17:425-431.
5. Baughman RP, Strohofer SA, Buchsbaum J, Lower EE. Release of tumor necrosis factor by alveolar macrophages of patients with sarcoidosis. J Lab Clin Med 1990;115:36-42.
6. Timmermans WM, van Laar JA, van Hagen PM, van Zelm MC. Immunopathogenesis of granulomas in chronic autoinflammatory diseases. Clin Transl Immunology 2016;5:e118.
7. Baughman RP, Drent M, Kavuru M, et al. Infliximab therapy in patients with chronic sarcoidosis and pulmonary involvement. Am J Respir Crit Care Med 2006;174:795-802.
8. Rossman MD, Newman LS, Baughman RP, et al. A double-blinded, randomized, placebo-controlled trial of infliximab in subjects with active pulmonary sarcoidosis. Sarcoidosis Vasc Diffuse Lung Dis 2006;23:201-208.
9. Hostettler KE, Studler U, Tamm M, Brutsche MH. Long-term treatment with infliximab in patients with sarcoidosis. Respiration 2012;83:218-224.
10. Cohen Aubart F, Bouvry D, Galanaud D, et al. Long-term outcomes of refractory neurosarcoidosis treated with infliximab. J Neurol 2017;264:891-897.
11. Gelfand JM, Bradshaw MJ, Stern BJ, et al. Infliximab for the treatment of CNS sarcoidosis: A multi-institutional series. Neurology 2017.
12. Zajicek JP. Neurosarcoidosis. Curr Opin Neurol 2000;13:323-325.
13. Tavee JO, Stern BJ. Neurosarcoidosis. Continuum (Minneap Minn) 2014;20:545-559.
14. Colombel JF, Sandborn WJ, Reinisch W, et al. Infliximab, azathioprine, or combination therapy for Crohn's disease. N Engl J Med 2010;362:1383-1395.
15. Rizzato G, Riboldi A, Imbimbo B, Torresin A, Milani S. The long-term efficacy and safety of two different corticosteroids in chronic sarcoidosis. Respir Med 1997;91:449-460.
16. Torres J, Boyapati RK, Kennedy NA, Louis E, Colombel JF, Satsangi J. Systematic Review of Effects of Withdrawal of Immunomodulators or Biologic Agents From Patients With Inflammatory Bowel Disease. Gastroenterology 2015;149:1716-1730.
17. Casanova MJ, Chaparro M, Garcia-Sanchez V, et al. Evolution After Anti-TNF Discontinuation in Patients With Inflammatory Bowel Disease: A Multicenter Long-Term Follow-Up Study. Am J Gastroenterol 2017;112:120-131.
18. Baert F, Noman M, Vermeire S, et al. Influence of immunogenicity on the long-term efficacy of infliximab in Crohn's disease. N Engl J Med 2003;348:601-608.
19. Ainsworth MA, Bendtzen K, Brynskov J. Tumor necrosis factor-alpha binding capacity and anti-infliximab antibodies measured by fluid-phase radioimmunoassays as predictors of clinical efficacy of infliximab in Crohn's disease. Am J Gastroenterol 2008;103:944-948.
20. Garces S, Demengeot J, Benito-Garcia E. The immunogenicity of anti-TNF therapy in immune-mediated inflammatory diseases: a systematic review of the literature with a meta-analysis. Ann Rheum Dis 2013;72:1947-1955.

21. Garcia-Vidal C, Rodriguez-Fernandez S, Teijon S, et al. Risk factors for opportunistic infections in infliximab-treated patients: the importance of screening in prevention. Eur J Clin Microbiol Infect Dis 2009;28:331-337.
22. Deepak P, Stobaugh DJ, Ehrenpreis ED. Infectious complications of TNF-alpha inhibitor monotherapy versus combination therapy with immunomodulators in inflammatory bowel disease: analysis of the Food and Drug Administration Adverse Event Reporting System. J Gastrointestin Liver Dis 2013;22:269-276.
23. Veltkamp M, Drent M, Baughman RP. Infliximab or biosimilars in sarcoidosis; to switch or not to switch? Sarcoidosis Vasc Diffuse Lung Dis 2016;32:280-283.
24. Schimmelpennink MC, Vorselaars ADM, van Beek FT, et al. Efficacy and safety of infliximab biosimilar Inflectra((R)) in severe sarcoidosis. Respir Med 2018;138S:S7-S13.
25. Yoo DH, Hrycaj P, Miranda P, et al. A randomised, double-blind, parallel-group study to demonstrate equivalence in efficacy and safety of CT-P13 compared with innovator infliximab when coadministered with methotrexate in patients with active rheumatoid arthritis: the PLANETRA study. Ann Rheum Dis 2013;72:1613-1620.
26. Jung YS, Park DI, Kim YH, et al. Efficacy and safety of CT-P13, a biosimilar of infliximab, in patients with inflammatory bowel disease: A retrospective multicenter study. J Gastroenterol Hepatol 2015;30:1705-1712.


Flinders Street
9

CHAPTER 6

GENERAL DISCUSSION

Parts of this chapter were published in
Clinical and Translational Immunology. 2016 Dec 16;5(12):e118
Sarcoidosis, Vasculitis and diffuse lung disease- 2017; 34; 269-271

The research described in this thesis focuses on granulomatous auto-inflammation. Granulomas are inflammatory structures with a complex interplay between macrophages, T cells and B cells. Typically, granulomas are formed in response to infections. For example, in patients with tuberculosis, the most prevalent infection worldwide, pulmonary granulomas are presumed an effective immunological response by encapsulating the tubercle bacillus. However, certain chronic immune-mediated non-infectious diseases are also characterized by granulomatous inflammation. The most well-known example is sarcoidosis, for which granuloma formation is the hallmark of disease. In other disorders, including Crohn's disease and common variable immunodeficiency (CVID), granulomas are present in substantial subsets of patients. In contrast to infectious causes, in which granulomas show signs of necrosis, granulomas in sarcoidosis and Crohn's disease do not usually have necrotic characteristics. Granuloma formation and maintenance in general, highly depends on the production of tumor necrosis factor alpha (TNF-α), explaining the effectivity of TNF-blocking therapy in both sarcoidosis and Crohn's disease. Infliximab is the most commonly used TNF-blocker in both diseases. We have aimed to study as described in this thesis, the immune compartment in granulomatous inflammatory diseases, with a focus on B cells in tissue and blood in order to improve diagnostics and identifying disease targets and markers for therapy.

Analysis of intestinal tissue in patients with Crohn's disease demonstrated numerous B cells around granulomas (Chapter 2). These patients had increased transitional B cells and anergic $CD21^{low}$ B cells in the peripheral blood. Furthermore, patients had fewer IgM memory B-cells and increased levels of somatic hypermutation (SHM) in IgA and IgG transcripts. In Chapter 3, we correlated the amount of B cells in the intestinal tissue with the presence of granulomas in Crohn's disease. With the addition of B-cell staining to the conventional hematoxylin and eosin (H&E) staining, the detection of granulomas was enhanced. Improved granuloma prevalence results in a better distinction between Crohn's disease and ulcerative colitis, another type of inflammatory bowel disease (IBD) with overlapping clinical features.

The observations in Chapter 2 and 3 on the relevance of B cells in Crohn's disease add to the B-cell aberrations that are seen in sarcoidosis. Whether these observations were clinically relevant was described in Chapter 4. B- and T-cells analysis before and during infliximab therapy demonstrated a rapid response in increase of numbers of IgM memory B cells, Tregs and Th17 T cells in patients who respond well to infliximab. Patients who developed anti-drug antibodies (ADA) during this study had normal levels of sIL-2r with lower levels of Tregs and higher levels of IgM memory B cells compared to responding patients at baseline. These observations indicate that sIL-2R, Tregs and IgM memory B cells may represent early markers for therapeutic success. More in depth B-cell analysis demonstrated increased levels of SHM in rearranged Ig transcripts persisted during therapy, indicating persistent chronic activation in this compartment. Remarkably, a

majority of the treated patients described in this chapter had an excellent response to infliximab. They represent sarcoidosis patients with a variety of organ involvement. The role of infliximab in neurosarcoidosis, an uncommon but often severe progressive type of sarcoidosis, was unclear with limited evidence from small number of case reports. In Chapter 5, results were presented from a multicenter retrospective cohort study of the effects of infliximab in neurosarcoidosis. Most of the patients showed a good response to infliximab with either improvement or remission of disease, however, there was a high chance of relapse after discontinuation. For the majority of patients who relapsed after discontinuation, a clinical response was present upon reintroduction of infliximab.

IMMUNOPATHOGENESIS

GRANULOMATOUS AUTO-INFLAMMATION

In Chapters 2 and 3, we demonstrated abundant B cells surrounding granulomas in Crohn's disease. Several studies have made similar observations in the past.(1) However, this was not actively pursued and both sarcoidosis and Crohn's disease were typically considered to be Th1 driven diseases. In more recent years, scientific opinion appears to be expanding for example, different types of T-cell subsets have been identified, and have been shown to be involved in these chronic immune-mediated diseases, such as Tregs and Th17 cells. Additionally, more research has been performed on the involvement of B cells.(2-5) The latter has become of interest as other immune-mediated diseases, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and granulomatosis with polyangiitis, display B-cell involvement. B-cell depletion in these disorders, for example with rituximab, generally results in clinical improvement.

In Chapters 2 and 3 of this thesis we have described that on top of $CD4^+$ T cells scattered in and around granulomas; a vast amount of B cells was seen around granulomas (Figure 1). B cells are typically known for their capacity to differentiate into plasma cells that produce antibodies. B cells also exhibit important other roles in the immune system, including antigen presentation, cytokine production, promoting a more Th1 or Th2 environment and B-T cell co-stimulation.(6) In Chapter 2 we showed that to a lesser extent, CD138 positive plasma cells were also present around granulomatous tissue, perhaps contributing to antibody production around granulomas. The question, therefore, is raised as to whether B cells contribute to a pro- or anti-inflammatory environment in granulomatous inflammation. B cells are also present around tuberculosis granulomas and in infected tissue.(7) It is hypothesized that further systemic damage is reduced by isolating the microbe within a granuloma.(7) However, in the case of chronic inflammatory diseases where no antigen can be truly identified, a microbiotic containment seems less relevant. Colitis murine models

underline the importance of B cells as these cells can suppress colitis resembling Crohn's disease by regulating T cells through IL-10 production,(8, 9) while in another model B cells prohibit regulatory T cells from suppressing T-cell function.(10) Costimulatory pathways including ICOS, CD40 and CD80 are involved in these murine models.(9-11) These data cannot be translated directly to human disease, yet it implies that the many B cells surrounding granulomas are not mere bystanders and may, therefore, have a more active role in this inflammatory state. This may be enhanced by the proximity to T cells, enabling B and T cells to interact. The exact role of B cells in granulomatous inflammation needs to be further studied, particularly due to the increased availability of treatments targeting B cells or B-T cell co-stimulatory pathways for immune-mediated diseases.

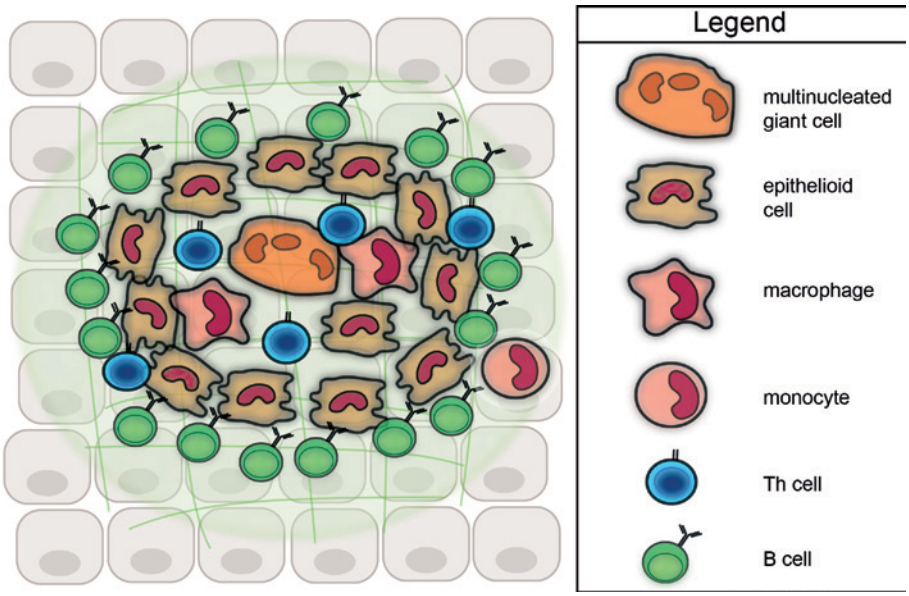


FIGURE 1. Model of the cellular organization of a non-caseating granuloma. Histology of granulomatous tissue (e.g. in Figure 1) displays the presence of macrophages, epithelioid cells and multinucleated giant cells in the core of the granuloma. Th cells are localized in and around the granuloma. B cells are rarely seen in granulomatous structures, however they are numerously present around granulomas.(3)

SYSTEMIC B-CELL ABNORMALITIES IN CROHN'S DISEASE AND SARCOIDOSIS

In Chapter 2, the peripheral blood compartment was extensively studied in patients with Crohn's diseases. This resulted in the identification of elevated levels of transitional and anergic $CD21^{low}$ B cells, while both $IgM^{+}IgD^{-}CD27^{+}$ 'IgM only' and $IgM^{+}IgD^{+}CD27^{+}$ 'natural effector' cells were decreased with normal absolute numbers of class-switched memory cells. The observed decrease in IgM memory B cells in Crohn's disease was in

line with previous studies, as was the increase in these cell numbers during infliximab therapy. (2, 12, 13) In addition to this, we showed that natural effector B cells in Crohn's disease might not be produced normally. Not only were IgM B-cell numbers decreased, but they also carried reduced SHM levels and their replication history was reduced and more in the range of naive B cells. In addition to decreased switched memory B cells, IgM memory B cells were also decreased in sarcoidosis, (3, 4, 14) and showed an increase in numbers during successful treatment with infliximab (Chapter 4). Interestingly, this finding extends to other immune-mediated diseases such as RA, SLE and spondyloarthritis.(15-17) In RA, natural effector B cells accumulate in the inflamed synovium of patients and traffic to the peripheral blood upon treatment with TNF-blockers.(15) As decreased IgM memory B cells are such a common finding throughout many immune-mediated diseases, it would be an interesting future research topic. For example, synovial inflamed tissue in RA, intestinal biopsies from colonoscopies in patients with Crohn's disease or skin biopsies of patients with skin sarcoidosis together with blood withdrawal from the same patient at the same time would represent the local and systemic inflammatory response. Through flowcytometric analysis, different lymphocytic subsets could be quantified and IgM memory B cells could be sorted for further analysis. IgM memory B cells in Crohn's disease showed less replications and SHM, but this has not been tested in any of the other diseases, let alone in IgM memory cells derived from inflamed tissue sites. One group explains the decrease in IgM memory B cells in Crohn's disease with decreased splenic function.(2) However, this seems less likely as another study showed low prevalence of hyposplenism in patients with Crohn's disease and the increased risk of infections with patients with Crohn's disease is mostly attributed to the use of immunosuppressive medications and not the disease itself since hyposplenism of decreased splenic function is seldomly described.(18, 19) Furthermore, only a subset of IgM memory B cells are derived from T-cell independent reactions and an estimated 67% is derived from germinal center reactions.(20, 21) Ideally, functional assays would be performed with the purified IgM-memory cells as, in general, function of these cells is still poorly understood. In healthy controls, IgM-memory B cells have higher expression of IFN-γ which can lead to an induction of class-switching to IgG2 and have the potential to re-enter germinal center reactions.(22) In both sarcoidosis and Crohn's disease we observed higher SHM levels and more IgG2 subclass usage, indicative of repetitive germinal center responses (Chapter 2 and 4). Perhaps these repetitive germinal center responses, with ongoing Ig class switching, could partly explain the lower level of IgM memory cells in peripheral blood. Future research on IgM memory cells in inflammatory disease, and specifically addressing their functional capacities, would be of significant interest as it could be translated to the function of IgM memory in healthy individuals and other immune-mediated diseases.

One noted difference in B-cell subsets between sarcoidosis and Crohn's disease is the distribution of $CD38^{dim}CD21^{low}$ B cells: these were increased in patients with Crohn's

whereas these numbers were normal in patients with sarcoidosis. These CD21^{low} B cells are an intriguing subset of cells because of the minimal occurrence in healthy controls. In contrast, increased levels were seen in patients with CVID, infections and autoimmunity.(20, 23-26) In general, these cells are functionally anergic and yet there is little understanding of the exact role of the function of CD21^{low} B cells. Interestingly, it was the only aberrant subset of B cells in our patients with Crohn's disease that did not normalize during treatment with infliximab suggesting that downregulation of CD21 is not affected by TNF-blockers. Furthermore, patients were treated with infliximab for a significant time (8 months to 10 years) and all had excellent therapeutic responses suggesting the expansion of CD21^{low} B cells is not related to a long-term therapeutic response. Further analysis of this subset would be of interest, particularly to study whether these cells show signs of normal apoptosis and exhaustion or have autoreactive B-cell receptors as these characteristics are described in other diseases. (24, 27, 28)

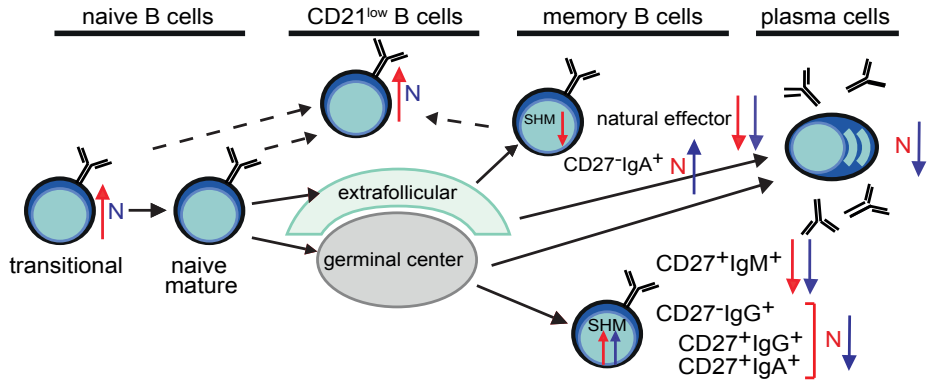


FIGURE 2. Characteristics of the peripheral B-cell compartment in Crohn's disease and sarcoidosis. Patients with Crohn's disease have elevated levels of transitional and CD21low B cells while normal in sarcoidosis. The IgM memory compartment is decreased in both diseases and in addition natural effector B cells were found to have decreased proliferation and SHM in Crohn's disease. Switched memory B cells on the other side, had normal values in Crohn's disease, yet were decreased in sarcoidosis. Interestingly, both diseases showed elevated levels of SHM in IgA en IgG transcripts. Red arrows represent results from Crohn's disease (Chapter 2); blue from sarcoidosis patients, derived from Kamphuis et al.(3) N; normal.

DIAGNOSTICS AND BIOMARKERS

The two most important types of IBD are Crohn's disease and ulcerative colitis, which differ in clinical symptoms, colonoscopy findings and histological features. However, due to the large overlap in symptoms and colonoscopy and histological findings there is currently no gold standard in how to diagnose and differentiate IBD. Diagnosis is considered a joint approach between clinical symptoms and features seen with

endoscopy and histology(29), however, this approach results in a change of diagnosis in up to 15% of patients during the course of disease and similar rates of patients being unclassified.(29)

The most discriminating histological feature is the presence of epithelioid granulomas.(30, 31) However, granulomas are only identified in 9-29% of biopsies of patients with Crohn's disease.(32-34) This is likely due to the fact that not all patients have granulomas, and due to sampling errors as the percentage of granulomas is higher in surgical resection samples of patients. However, granulomas in Crohn's disease can be very small (microgranulomas) and are therefore difficult to detect. We identified the presence of many B cells, specifically around granulomatous tissue in patients with Crohn's disease, while B cells were not present in non-granulomatous inflammatory tissue of patients with Crohn's disease (chapter 2). We hypothesized that by focussing on B cells in inflamed tissue of IBD patients, it might be possible to increase the yield of granulomas and thereby identify true Crohn's disease patients. Indeed in Chapter 3, we demonstrated that dense B-cell infiltrates were indicative of granuloma formation. In this retrospective study, the addition of CD20 staining also resulted in a higher prevalence of granulomas. Therefore, CD20 staining could be a logical addition to the currently used H&E staining in order to optimize the diagnostic process of IBD. This is of clinical relevance as disease management is increasingly based on disease specific regimens. For example, methotrexate is only used to treat Crohn's disease, while ileal-pouch-anal anastomosis surgery is preferred in patients with ulcerative colitis, or in a highly selected group of patients with Crohn's disease.(29, 35) Moreover, recent studies with vedolizumab, a biological inhibiting the adhesion of lymphocytes to the endothelial cells in the intestinal tract, showed that this compound was more effective in ulcerative colitis than in Crohn's disease. This indicates that both entities differ not only in immunopathogenesis, but also in therapeutic approach. Those findings strengthen the value for optimal clinical differentiation for example by adding a simple CD20 staining in the histological samples.(36, 37) The increasing availability of new biologicals will prompt, even more so, the need for a correct diagnosis in IBD patients in order to direct disease-specific targeted therapy (precision medicine). The results of our retrospective study are merely a first step into optimizing diagnostics with CD20 staining. A prospective trial with a larger cohort of newly-diagnosed IBD patients would be necessary to identify whether adding to the diagnostic process for IBD would positively impact daily practice. In Chapter 3, we were only able to analyze one biopsy site and one slide per patient. Hence, there was a substantial risk of sampling error, and the rate of granuloma detection would most likely be further increased by analysis of slides from multiple biopsy locations. CD20 is a pan B-cell marker which is used on a routine basis as a diagnostic tool for haematological malignancies.(38) This staining is relatively straight-forward and inexpensive, and can be implemented easily into the clinical work-up. It would be interesting to combine this method with analyzing anti-Saccharomyces cerevisiae antibodies, which are present

in the majority of patients with Crohn's disease, but not in ulcerative colitis, and could support differential diagnosis between the two disease entities.(39) Potentially, the described combined approach will lead to a decrease of misdiagnosis in IBD patients of 9%.(40) More refined diagnostics will become of greater importance as many new disease specific drugs and targeted therapies are currently being trialled or are expected on the market in the immediate future.(41)

TREATMENT MONITORING

With the increasing variations and opportunities for disease-specific targeted biological therapies, it has become increasingly important to appropriately select patients who are likely to benefit from a targeted therapy. While several agents can be efficacious in patients, there are still subgroups of patients with refractory disease.(42, 43) For example, only 60% of patients with Crohn's disease achieve disease control within 26 weeks.(44) Starting a patient on ineffective therapy can be expensive and will delay the start of a potentially effective treatment with the additional risk of disease exacerbation and subsequent morbidity. Therapeutic drug monitoring for infliximab has been extensively studied in both Crohn's disease and RA.(45, 46) Serum infliximab trough levels have become a standard monitoring tool, because low drug levels are indicative of the formation of antibodies against infliximab, ADA that can hamper therapy success.(47) In contrast to RA and Crohn's disease, both optimal trough levels of infliximab and the formation frequency of ADAs in patients with sarcoidosis had not been thoroughly examined. Chapter 4 described a mean serum infliximab trough level of 4.6 µg/mL with 4 out of 10 patients in this study with a level below the 3 µg/mL threshold. However, only one these patients relapsed at that time-point with nearly undetectable infliximab levels together with ADA, whereas the other three patients had infliximab levels between 2 and 3 µg/mL without formation of ADA. Furthermore, 2 out these 3 patients showed a long-term response (several years) to infliximab. Infliximab trough levels below 3 µg/mL in patients with a successful long-term therapeutic response raise the question whether the optimal infliximab levels might be disease-specific and therefore may be different in patients with sarcoidosis, or whether serum trough levels do not necessarily reflect tissue levels. Infliximab tissue levels have not been well studied in general, yet patients with Crohn's disease only have higher tissue levels of inflamed and non-inflamed tissue when serum trough levels were also higher which is associated with long-term response.(48) So far, in two studies with sarcoidosis patients, mean trough infliximab levels ranged between 7.5 and 18 µg/mL.(43, 49) Furthermore, optimizing therapy can be cost-reductive in these rather costly biological therapies and therefore this should be further studied in sarcoidosis, especially in relation to ADA, optimal serum trough levels and tissue levels.

Currently, serum sIL-2R levels are most widely used as a biomarker in sarcoidosis as these seem to reflect disease activity better than angiotensin converting enzyme (ACE) levels.(50, 51) The relevance of sIL-2R as a biomarker is further stressed in Chapter 4, in which we show that 9 out of 11 patients had elevated levels before the start of infliximab treatment with a subsequent decrease following a good response to infliximab. Interestingly, the 2 patients who initially presented with sIL-2R levels within the normal range did not have a good therapeutic response with detectable ADA within 8 months of treatment. These observations suggest that patients with active disease, here reflected by higher sIL-2R levels, most likely benefit most from infliximab. To our knowledge, only one small study (n=11 patients) specifically analyzed sIL-2R in neurosarcoidosis, and concluded it to be a valuable marker of disease for such patients.(52) The results in Chapter 5 confirm sIL-2R as a useful biomarker in neurosarcoidosis with the majority of patients (9/14; 64%) showing normalization of levels during infliximab treatment. Therefore, it can be recommended for sIL-2R to be routinely measured in patients with both neurosarcoidosis and sarcoidosis.

Another biomarker that has been suggested to monitor therapeutic efficacy in sarcoidosis is B-cell activating factor (BAFF). Serum BAFF levels are elevated in patients with chronic active sarcoidosis.(14, 53) However, these results were not confirmed by the follow-up study with infliximab in this thesis (Chapter 4). The serum BAFF levels did not differ significantly between the control and patient groups. While there were some patients with very high levels, the BAFF levels in many patients were within the range of controls. In addition, no change was seen during infliximab therapy, indicating that BAFF is less suitable as a biomarker or monitoring tool in sarcoidosis.

Other suggested biomarkers include sCD163.(54) Indeed, in preliminary analysis patients with active sarcoidosis had significantly higher levels of soluble (sCD163) than healthy controls (Figure 3, unpublished data). These levels were decreased in week 6 of infliximab therapy. However, despite the significant difference between groups, there was a large overlap in levels from patients and controls. sCD163 levels at baseline correlated significantly with sIL-2R at baseline ($p=0.04$), and this correlation nearly reached significance at month 8 ($p=0.07$). CD163 expression in sarcoidosis biopsies was significantly increased compared to tuberculosis samples, suggesting a polarization of macrophages to the M2 phenotype which is generally associated with chronicity towards a fibrotic state in other studies.(55) In accordance to this data, sCD163 was also found to be elevated in the serum of sarcoidosis patients with active disease, suggesting this assay could be used as a biomarker.(54, 55) However, the overlap in levels between controls and patients in our study indicate that the sole use of sCD163 as a biomarker will not suffice and a combination with other markers might be needed to increase sensitivity.

In this thesis, we demonstrated differences in specific lymphocyte subsets between patients with active sarcoidosis and controls (Chapter 4). Since these subsets can be

readily detected by flowcytometry, such measurements could be incorporated in daily practice to monitor disease activity and therapeutic efficacy. Numbers of IgM memory B cells, Tregs and Th17 cells are candidate tools. To date, lymphocyte subset analysis is rarely used in other immune-mediated diseases as reproducibility appears to hamper accurate results for example, due to differences in patient groups or differences in sample processing procedures.(56) For now, the results presented in this thesis identify key areas in the inflammatory processes ongoing in immune-mediated diseases. In the near future, when distinct patterns are identified in larger cohorts, the potential use of subsets in a more standardized and applicable manner could be more easily applied to routine clinical practice.

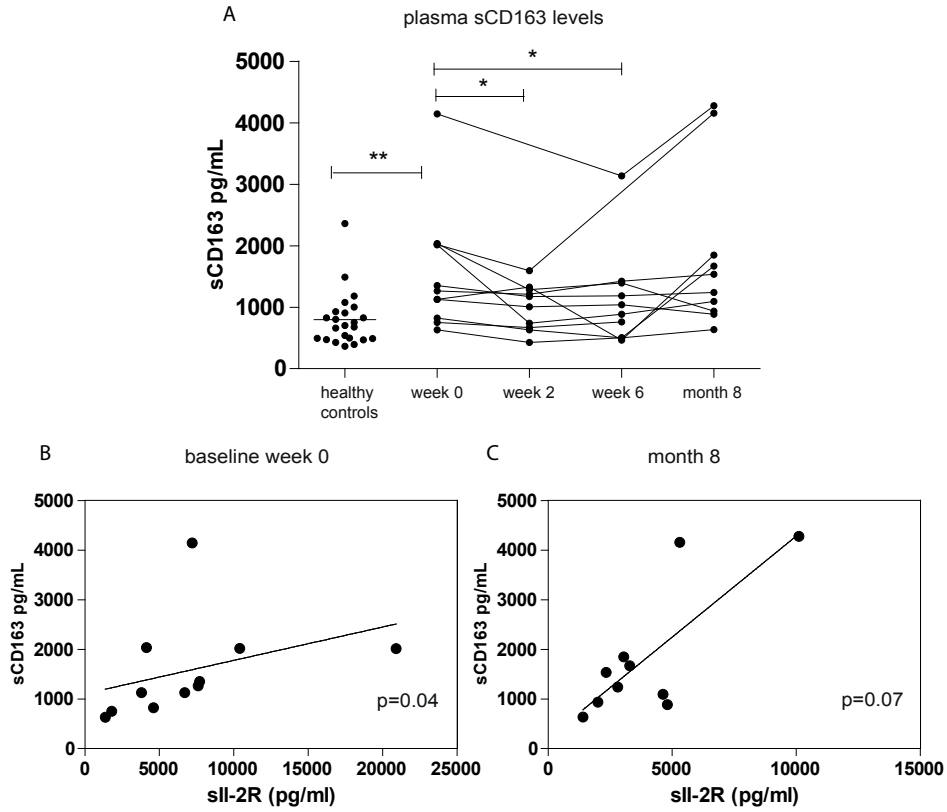


FIGURE 3. Scatter-dot plot of plasma sCD163 levels during infliximab treatment in patients with sarcoidosis and correlation with sIL-2R at baseline (B) and month 8 (C) of therapy. Statistical analysis includes paired Mann-Whitney test (A) and Spearman correlation analyzations (B and C); patients and controls are from Chapter 4.
*, P<0.05; **, P<0.01.

THERAPY

In this thesis we showed clinical efficacy of infliximab in pulmonary and extra-pulmonary sarcoidosis (Chapter 4) and neurosarcoidosis (Chapter 5). The initial randomized controlled trial (RCT) from Baughman *et al*. in pulmonary sarcoidosis only identified a minor effect of mean increase in FVC of 2.5%.(43) However, subsequent studies identified more positive outcomes with an overall disease improvement ranging from 60-92%. (49, 57-59) Our own experiences in extra-pulmonary sarcoidosis have prompted the use of infliximab in patients with sarcoidosis in daily practise as a third line therapy, and as induction therapy in some instances such as neuro-sarcoidosis or vision-threatening sarcoid-induced uveitis. The positive effects described in Chapter 4 and 5 might be explained by including patients with active and serious sarcoidosis reflected by the high levels of sIL-2R and/or positive nuclear scans. Furthermore, such patients with active and organ-threatening disease have high levels of TNF-α and represent a cohort that has been associated to benefit most from TNF-blocking therapy in other studies with sarcoidosis patients.(49, 60) The data presented in this thesis emphasizes the need for follow-up studies with designs that better reflect daily clinical practice. The data of patients with neurosarcoidosis studied in Chapter 5 also illustrates that, in spite of overall high response rates with improvement or remission of disease, patients often show a relapse of disease after discontinuation of therapy. In line with data from Crohn's disease, patients who were reintroduced to infliximab often remain responsive. This data indicate that infliximab can be safely discontinued when a long and stable period of remission is achieved.(61) The exact duration of that response needs to be determined.

Recently, biosimilars of infliximab have become available.(62) Since 2016, our center (and many others) treat all patients with a biosimilar (Remsima). Patients enrolled in the studies presented in Chapter 4 and 5, were started on therapy in 2016 or 2017, and were therefore treated with Remsima. Large studies in RA and Crohn's disease have shown that efficacy was similar to the original Remicade, while being cost-reductive. (63, 64) Recently, the safety and effectivity of biosimilars has also been demonstrated for sarcoidosis.(65) In addition to the clinical effectivity of infliximab biosimilars, the immunogenicity of biosimilar Remsima was similar to that of the original Remicade, as reflected by the infliximab trough levels and ADA assays in Chapter 4. The ability to prescribe biosimilars and therefore reducing the overall costs of health care, is of particular interests in patients with sarcoidosis as it is a rare disease with off-label restrictions.

OCTREOTIDE (SANDOSTATIN©) THERAPY IN PATIENTS WITH CHRONIC SARCOIDOSIS

Many patients with chronic sarcoidosis face side effects of treatment and refractory disease. Therefore, there is still a need for new therapeutic options, and the search for novel treatment strategies is warranted. A better understanding of the immune-

pathophysiology and cellular and molecular characteristics of specific diseases could help to identify targets for new therapeutic agents.

Octreotide is one of these drug candidates. Treatment with this somatostatin (SS) analogue has demonstrated significant therapeutic effects in patients with in cystoid macula edema, neuro-endocrine tumors (NET) and various other diseases.(66) Octreotide is thought to exerts its therapeutic effect in NET by binding to somatostatin receptors sst2 and sst5 in SS-positive lesions. As a result, the hormone secretion from the tumor is inhibited resulting in apoptosis and cell cycle arrest.(67) Sst2 is widely expressed by monocytes and macrophages in steady state as well as by monocytes, macrophages and epithelioid cells in granulomatous tissue, e.g. in sarcoidosis.(68, 69) Somatostatin receptor scintigraphy (SRS) is a valuable tool in diagnosis of sarcoidosis through detection of sst2 positive lesions with imaging.(70, 71) Therefore, it can be hypothesized that by inhibiting sst2 with octreotide the activity of sst2 positive sarcoid lesions can be influenced similarly to those of NET. Indeed, two sarcoidosis patients have been treated with octreotide.(69) One patient with pulmonary and mediastinal involvement did not show a clinical response, while the other patient with refractory skin and lymph node sarcoidosis showed a decrease in SRS uptake and remission of skin lesions and lymph node size.(69) Both patients had biopsies positive for sst2 and positive SRS, yet only one patient showed a clinical response to octreotide.

We therefore performed a preliminary analysis of the clinical effects of Sandostatin, a long-acting octreotide preparation, in two SRS positive chronic sarcoidosis patients in a prospective Phase II study (MEC-2014-099, NTR4655). Included patients had biopsy-proven, SRS positive chronic sarcoidosis for >3 years with indication for therapy with involvement of skin, joint, lymph node and/or lung (diffusion capacity between 60-75%) (Table 1). The primary endpoint of this study was change in uptake per organ measured with the four point scale as previous described.(71) The secondary endpoint included blood tests, RAND-36 score and pulmonary function test. Due to the limited number of enrolled patients in this study, we have also reported the results of our two therapy-refractory patients treated with octreotide outside this trial.

Uptake on SRS did not change during treatment (Table 1). In addition, no substantial or durable improvement was seen in secondary endpoints including pulmonary function and sIL-2R. Furthermore, clinical evaluation did not show a decrease in disease activity. In this respect our patients still showed symptoms in spite of Sandostatin; patient 1 had evident arthritis, patient 2 experienced general systemic complaints, while patient 3 had a uveitis flare and patient's 4 kidney function continued to decrease.

The adverse events caused by Sandostatin are well known due to its extensive use in other diseases. Most common side effects of Sandostatin are gastrointestinal complaints; nausea and diarrhea, these side-effects usually show spontaneous resolution.(66) All patients reported mild gastrointestinal complaints as described in literature. Patient 1 developed a significant increase in liver enzymes of more than 3 times the normal values.

TABLE 1. Preliminary results of primary and secondary endpoints at baseline and during treatment with sandostatin

Patient/ Gender/ Age	Disease duration (y)/ sandostatin duration (m)	Trial yes/no	Indication	Initial SRS score	SRS score	Initial FVC (%)	FVC (%)	Initial DLCO (%)	DLCO (%)	Initial Eye activity	Eye activity	Initial sIL-2R	sIL-2R	Initial Creatinine	Creatinine	Efficacy yes/no
1/M/43	11/4	yes	Joints	4	ND	111	117	83	ND	ND	ND	7300	6100	ND	ND	no
2/F/55	21/6	yes	Pulmonary	4	5	81	83	62	63	ND	ND	2500	2100	ND	ND	no
3/F/50	7/11	no	Uveitis	ND	ND	112	110	45	41	uveitis	Flare uveitis*	16900	10400	ND	ND	no
4/F/58	10/5	no	Kidney	6	6	ND	ND	ND	ND	ND	ND	28900	38600	160	173	no

Results indicated before start (initial) and during treatment with sandostatin. Pulmonary functions FVC and DLCO presented in % predicted. * Patient 3 had a flare of panuveitis of the left eye with a need of starting corticosteroid treatment. sIL-2R in pg/mL; creatinine in µmol/L. M; Male, F; Female, y; years, m; months, SRS; somatostatin receptor scintigraphy, ND; not determined, FVC; forced vital capacity, DLCO; diffusing capacity, sIL-2R; soluble interleukin-2 receptor.

Sandostatin was discontinued whereupon the enzymes quickly decreased to a stable level as they had been prior to the study and the patient chose to discontinue with the study. Additionally, gallstones can be formed and have been reported in 20-30% of patients, although in most cases these are asymptomatic and therefore do not lead to any significant events.(66) Patient 2 developed symptomatic gallstones after 11 months leading to a cholecystectomy. Patient 3 had significant weight loss during treatment necessitating stop of treatment. Patient 4 discontinued sandostatin after 5 months due to lack of effectivity. In conclusion, we did not observe a response on SRS or other any clinical parameter. Adversely, our patients did experience gastrointestinal adverse events including one serious adverse event; cholelithiasis leading to cholecystectomy.

Unfortunately, the negative results from the Sandostatin trial in sarcoidosis, are not the only failed study in sarcoidosis. For example, studies with etanercept and golimumab, other TNF-blockers and ustekinumab, anti p40 targeting the IL12/IL23 pathway reported negative results.(72, 73) All these studies included patients with chronic, stable disease with a stable dose of corticosteroids, which may contribute to the minimal benefits reported. The infliximab RCT in a similar cohort of sarcoidosis patients also demonstrated, at best, a very modest result, raising doubts over the clinical significance.(43) However, these discreet results have not prevented others from using infliximab and it is now widely accepted as a clinical therapeutic agent for many patients with sarcoidosis.(43, 57) The results suggest a differential response to infliximab with patients with less active disease and pre-existent fibrosis. Infliximab is not registered for the use of sarcoidosis and is used off-label, resulting in challenges and restrictions in prescribing this costly agent. Interestingly, the results coming from the use of infliximab in daily practice in patients with active and severe sarcoidosis are better than the RCT, as shown in Chapter 4 and 5. Therefore, it is necessary to organize well-structured trials to determine if patients with biopsy proven and active sarcoidosis can truly benefit from new treatment strategies.

POTENTIAL FUTURE TARGETS FOR TREATMENT OF GRANULOMATOUS DISEASES

New therapies are in high demand for refractory patients with chronic inflammatory disorders. As new therapeutic targets become evident and new biologicals may become available, even for patients with granulomatous disorders, there are several therapeutic candidates involving the B- and/or T cells (Figure 4).

IL-21 is a cytokine produced by Th cells and stimulates B and T cells through the IL-21 receptor. Increased levels of IL-21 were seen in inflamed tissue of Crohn's disease patients with infliximab inducing a downregulation of IL-21.(74) IL-21 is also implicated in the immunopathogenesis of RA and therefore treatment with a new monoclonal antibody binding to IL-21, NNC0114-0005, is currently being tested in a clinical trial.

(75) When safety and efficacy is proven in RA, this treatment could be translated into other inflammatory disorders such as Crohn's disease. However, caution should be taken because genetic defects in IL-21 were recently reported to cause a severe CVID-like disorder,(76) and early onset IBD.(77)

Interestingly, T-cell function might be improved through targeting of the inhibitory receptor programmed death-1 (PD-1).(78) In patients with sarcoidosis, PDL-1 expression is increased on T cells in granulomatous tissue, and numbers of PD1-expressing Th cells in blood are increased.(79) As a downregulation of PD-1 on CD4 cells was seen in patients with spontaneous clinical resolution, blocking the PD-1/PD-1L pathway could be a therapeutic target.(79) A variety of malignancies also show upregulation of the PD-1/PD-1L pathway and currently several antibodies against PD-1, such as pembrolizumab and nivolumab, are being tested in treatment of solid and haematological malignancies.(80) Still, a cautious approach is warranted as sarcoidosis-induced disease has also been reported with the use of pembrolizumab in a patient with sarcoma.(81) This case could be explained by the enhanced CD4 T-cell proliferation that was reported when PD-1 was blocked, which could lead to a Th1 pro-inflammatory response that is also observed in sarcoidosis.

Inducible T-cell COStimulator (ICOS) and ICOSL are important factors in adaptive immunity through B-T cell interaction and genetic defects in ICOS have been reported to result in adult onset CVID.(82) Moreover, *ICOSL* gene polymorphisms are associated with Crohn's disease,(83) and increased ICOS expression was reported on Tregs in patients with sarcoidosis.(84) A monoclonal antibody targeting ICOSL, AMG-557, has been developed and is currently undergoing the first trial in SLE. While treatment targeting the ICOS/ICOSL pathway is still under development, it is a potential target of interest for granulomatous inflammatory diseases, because ICOS/ICOSL might be involved in the immunopathophysiology of both sarcoidosis and Crohn's disease. Moreover, targeting this co-stimulatory pathway affects both T- and B-cell activation without their cellular depletion.(85)

Finally, promising therapeutics offering alternative approaches to target B cells in addition to CD20 blocking agents are in development. Currently, belimumab, a monoclonal antibody targeting BAFF has been implemented in the treatment of SLE. BAFF is a cytokine produced mainly by macrophages, and it is essential for mature B-cell survival.(86) Autoreactive B cells are especially dependent on high BAFF levels.(87) Patients with active sarcoidosis, Crohn's disease and CVID can display increased BAFF levels.(14, 53, 88, 89) Furthermore, BAFF promotes a Th17 response and produces TNF-α, both of which are involved in these diseases. Belimumab may, therefore, be an interesting candidate as not only pathogenic B cells are targeted, but also the complex interplay between T cells and pro-inflammatory cytokines is covered.(86, 90, 91)

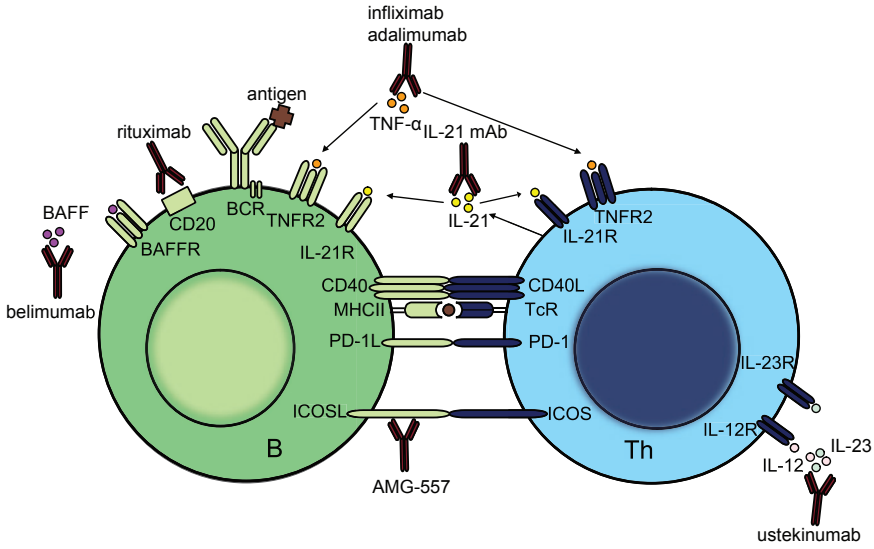


FIGURE 4. Novel therapeutics that are currently used or in trial for treatment of granulomatous autoinflammatory diseases that target B- and/or T cells. Indicated are monoclonal antibodies that specifically target T cells (ustekinumab), or B cells (rituximab and belimumab). Anti-TNFα (infliximab, adalimumab) and anti-IL-21 monoclonal antibodies block cytokines that affect both B and T cells. Finally, AMG-557 targets ICOSL and affects the B-T cell interaction: BCR, B-cell receptor; mAb, monoclonal antibody; MHCII, major histocompatibility complex class 2; TcR, T-cell receptor.

CONCLUDING REMARKS

Granulomatous inflammation seen in sarcoidosis and Crohn's disease is a complex interplay between mature macrophages, Th cells and B cells. In this thesis we demonstrate, with in depth B-cell analyses, that B-cells are indeed involved in the inflammatory process of granuloma formation. The most important findings that B-cells are involved in granuloma formation/homeostasis in this thesis are: 1) the B-cell compartment in patients with Crohn's disease shows dysregulation particularly in the IgM-memory compartment with decreased IgM memory cells accompanied by decreased number of SHM and fewer cell divisions. 2) B cells are numerously present around granulomas in Crohn's disease. 3) numbers of IgM memory B cells correlate with a therapeutic response to infliximab in patients with sarcoidosis and Crohn's disease. These findings implicate that B cells are not only vastly present in granulomatous inflammation, they have an active role in this process, possibly through B-T cell interaction. Our understanding of the immune system is improving, which has led to the development of targeted, molecular designed agents (biologicals) of which the TNF-α blockers are the most studied and successful examples. In this thesis, we describe the results of infliximab beyond the environment of a randomized controlled trial, in patients with different types of sarcoidosis including pulmonary sarcoidosis and neurosarcoidosis. These

observations demonstrated better therapeutic responses than initially described in the randomized controlled trial. Therefore, infliximab seems to be an effective therapeutic agent in, yet to be determined, subgroups of patients with sarcoidosis. Such subgroups might include patients with active, extra pulmonary but also pulmonary sarcoidosis (Chapter 4 of this thesis) and neurosarcoidosis (Chapter 5). To further enhance the success rate, infliximab could possibly be further optimized by 1) treating only patients with severe and active sarcoidosis; 2) co-medication with methotrexate or azathioprine to reduce development of ADA; 3) therapy monitoring through sIL-2R and infliximab levels, combined with blood B- and T-cell analysis.

The complexity of treating chronic inflammatory diseases is illustrated by numerous failed drug trials, while the need for new therapeutics is needed due to the substantial occurrence of refractory disease. A translational approach and implementing advances in other immune-mediated diseases remains necessary to improve treatment options for these refractory patients. Studies into granuloma formation in genetically-defined immunodeficiencies can provide candidate pathways, whereas insights into immune dysregulation in sarcoidosis and Crohn's disease can provide immunological markers to identify CVID patients at risk for granulomatous complications. Recent insights into disease pathogenesis and the potential involvement of B cells open new avenues for treatment and, in particular, patients with granulomatous inflammatory disease may benefit from targeting B cells or B-T cell interactions with new therapeutics.

REFERENCES

1. Geboes K, van den Oord J, De Wolf-Peeters C, et al. The cellular composition of granulomas in mesenteric lymph nodes from patients with Crohn's disease. Virchows Arch A Pathol Anat Histopathol 1986;409:679-92.
2. Di Sabatino A, Rosado MM, Ciccocioppo R, et al. Depletion of immunoglobulin M memory B cells is associated with splenic hypofunction in inflammatory bowel disease. Am J Gastroenterol 2005;100:1788-95.
3. Kamphuis LS, van Zelm MC, Lam KH, et al. Perigranuloma localization and abnormal maturation of B cells: emerging key players in sarcoidosis? Am J Respir Crit Care Med 2013;187:406-16.
4. Lee NS, Barber L, Akula SM, et al. Disturbed homeostasis and multiple signaling defects in the peripheral blood B-cell compartment of patients with severe chronic sarcoidosis. Clin Vaccine Immunol 2011;18:1306-16.
5. Li Z, Vermeire S, Bullens D, et al. Anti-Tumor Necrosis Factor Therapy Restores Peripheral Blood B-cell Subsets and CD40 Expression in Inflammatory Bowel Diseases. Inflamm Bowel Dis 2015;21:2787-96.
6. LeBien TW, Tedder TF. B lymphocytes: how they develop and function. Blood 2008;112:1570-80.
7. Gonzalez-Juarrero M, Turner OC, Turner J, et al. Temporal and spatial arrangement of lymphocytes within lung granulomas induced by aerosol infection with Mycobacterium tuberculosis. Infect Immun 2001;69:1722-8.
8. Mizoguchi E, Mizoguchi A, Preffer FI, et al. Regulatory role of mature B cells in a murine model of inflammatory bowel disease. Int Immunol 2000;12:597-605.
9. Mizoguchi A, Mizoguchi E, Takedatsu H, et al. Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation. Immunity 2002;16:219-30.
10. Olson TS, Bamias G, Naganuma M, et al. Expanded B cell population blocks regulatory T cells and exacerbates ileitis in a murine model of Crohn disease. J Clin Invest 2004;114:389-98.
11. Eri R, Kodumudi KN, Summerlin DJ, et al. Suppression of colon inflammation by CD80 blockade: evaluation in two murine models of inflammatory bowel disease. Inflamm Bowel Dis 2008;14:458-70.
12. Di Sabatino A, Rosado MM, Cazzola P, et al. Splenic function and IgM-memory B cells in Crohn's disease patients treated with infliximab. Inflamm Bowel Dis 2008;14:591-6.
13. Li Z, Vermeire S, Bullens D, et al. Restoration of Foxp3+ Regulatory T-cell Subsets and Foxp3- Type 1 Regulatory-like T Cells in Inflammatory Bowel Diseases During Anti-tumor Necrosis Factor Therapy. Inflamm Bowel Dis 2015;21:2418-28.
14. Saussine A, Tazi A, Feuillet S, et al. Active chronic sarcoidosis is characterized by increased transitional blood B cells, increased IL-10-producing regulatory B cells and high BAFF levels. PLoS One 2012;7:e43588.
15. Souto-Carneiro MM, Mahadevan V, Takada K, et al. Alterations in peripheral blood memory B cells in patients with active rheumatoid arthritis are dependent on the action of tumour necrosis factor. Arthritis Res Ther 2009;11:R84.
16. Salinas GF, De Rycke L, Barendregt B, et al. Anti-TNF treatment blocks the induction of T cell-dependent humoral responses. Ann Rheum Dis 2013;72:1037-43.
17. Rodriguez-Bayona B, Ramos-Amaya A, Perez-Venegas JJ, et al. Decreased frequency and activated phenotype of blood CD27 IgD IgM B lymphocytes is a permanent abnormality in systemic lupus erythematosus patients. Arthritis Res Ther 2010;12:R108.

18. Muller AF, Cornford E, Toghill PJ. Splenic function in inflammatory bowel disease: assessment by differential interference microscopy and splenic ultrasound. Q J Med 1993;86:333-40.
19. Toruner M, Loftus EV, Jr., Harmsen WS, et al. Risk factors for opportunistic infections in patients with inflammatory bowel disease. Gastroenterology 2008;134:929-36.
20. Berkowska MA, Driessen GJ, Bikos V, et al. Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways. Blood 2011;118:2150-8.
21. Bagnara D, Squillario M, Kipling D, et al. A Reassessment of IgM Memory Subsets in Humans. J Immunol 2015;195:3716-24.
22. Seifert M, Przekopowitz M, Taudien S, et al. Functional capacities of human IgM memory B cells in early inflammatory responses and secondary germinal center reactions. Proc Natl Acad Sci U S A 2015;112:E546-55.
23. Isnardi I, Ng YS, Menard L, et al. Complement receptor 2/CD21- human naive B cells contain mostly autoreactive unresponsive clones. Blood 2010;115:5026-36.
24. Moir S, Malaspina A, Ogwaro KM, et al. HIV-1 induces phenotypic and functional perturbations of B cells in chronically infected individuals. Proc Natl Acad Sci U S A 2001;98:10362-7.
25. Wehr C, Eibel H, Masilamani M, et al. A new CD21low B cell population in the peripheral blood of patients with SLE. Clin Immunol 2004;113:161-71.
26. Rakhmanov M, Gutenberger S, Keller B, et al. CD21low B cells in common variable immunodeficiency do not show defects in receptor editing, but resemble tissue-like memory B cells. Blood 2010;116:3682-3.
27. Glauzy S, Boccitto M, Bannock JM, et al. Accumulation of Antigen-Driven Lymphoproliferations in Complement Receptor 2/CD21(-/low) B Cells From Patients With Sjogren's Syndrome. Arthritis Rheumatol 2018;70:298-307.
28. Del Padre M, Todi L, Mitrevski M, et al. Reversion of anergy signatures in clonal CD21(low) B cells of mixed cryoglobulinemia after clearance of HCV viremia. Blood 2017;130:35-38.
29. Tontini GE, Vecchi M, Pastorelli L, et al. Differential diagnosis in inflammatory bowel disease colitis: state of the art and future perspectives. World J Gastroenterol 2015;21:21-46.
30. Le Berre N, Heresbach D, Kerbaol M, et al. Histological discrimination of idiopathic inflammatory bowel disease from other types of colitis. J Clin Pathol 1995;48:749-53.
31. Seldenrijk CA, Morson BC, Meuwissen SG, et al. Histopathological evaluation of colonic mucosal biopsy specimens in chronic inflammatory bowel disease: diagnostic implications. Gut 1991;32:1514-20.
32. Freeman HJ. Granuloma-positive Crohn's disease. Can J Gastroenterol 2007;21:583-7.
33. Heresbach D, Alexandre JL, Branger B, et al. Frequency and significance of granulomas in a cohort of incident cases of Crohn's disease. Gut 2005;54:215-22.
34. Turner K, Genta RM, Lujan G, et al. Significance of the epithelioid granuloma in biopsies of Crohn's colitis. Inflamm Bowel Dis 2014;20:2271-5.
35. Chang S, Shen B, Remzi F. When Not to Pouch: Important Considerations for Patient Selection for Ileal Pouch-Anal Anastomosis. Gastroenterol Hepatol (N Y) 2017;13:466-475.
36. Cominelli F. Inhibition of leukocyte trafficking in inflammatory bowel disease. N Engl J Med 2013;369:775-6.
37. Feagan BG, Rutgeerts P, Sands BE, et al. Vedolizumab as induction and maintenance therapy for ulcerative colitis. N Engl J Med 2013;369:699-710.
38. Wang HY, Zu Y. Diagnostic Algorithm of Common Mature B-Cell Lymphomas by Immunohistochemistry. Arch Pathol Lab Med 2017;141:1236-1246.

39. Quinton JF, Sendid B, Reumaux D, et al. Anti-Saccharomyces cerevisiae mannan antibodies combined with antineutrophil cytoplasmic autoantibodies in inflammatory bowel disease: prevalence and diagnostic role. Gut 1998;42:788-91.
40. Moss AC, Cheifetz AS. How often is a diagnosis of ulcerative colitis changed to Crohn's disease and vice versa? Inflamm Bowel Dis 2008;14 Suppl 2:S155-6.
41. Catalan-Serra I, Brenna O. Immunotherapy in inflammatory bowel disease: Novel and emerging treatments. Hum Vaccin Immunother 2018:1-15.
42. Hanauer SB, Feagan BG, Lichtenstein GR, et al. Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. Lancet 2002;359:1541-9.
43. Baughman RP, Drent M, Kavuru M, et al. Infliximab therapy in patients with chronic sarcoidosis and pulmonary involvement. Am J Respir Crit Care Med 2006;174:795-802.
44. Colombel JF, Sandborn WJ, Reinisch W, et al. Infliximab, azathioprine, or combination therapy for Crohn's disease. N Engl J Med 2010;362:1383-95.
45. Ainsworth MA, Bendtzen K, Brynskov J. Tumor necrosis factor-alpha binding capacity and anti-infliximab antibodies measured by fluid-phase radioimmunoassays as predictors of clinical efficacy of infliximab in Crohn's disease. Am J Gastroenterol 2008;103:944-8.
46. Bendtzen K, Geborek P, Svenson M, et al. Individualized monitoring of drug bioavailability and immunogenicity in rheumatoid arthritis patients treated with the tumor necrosis factor alpha inhibitor infliximab. Arthritis Rheum 2006;54:3782-9.
47. Baert F, Noman M, Vermeire S, et al. Influence of immunogenicity on the long-term efficacy of infliximab in Crohn's disease. N Engl J Med 2003;348:601-8.
48. Yoshihara T, Shinzaki S, Kawai S, et al. Tissue Drug Concentrations of Anti-tumor Necrosis Factor Agents Are Associated with the Long-term Outcome of Patients with Crohn's Disease. Inflamm Bowel Dis 2017;23:2172-2179.
49. Vorselaars AD, Crommelin HA, Deneer VH, et al. Effectiveness of infliximab in refractory FDG PET-positive sarcoidosis. Eur Respir J 2015;46:175-85.
50. Grutters JC, Fellrath JM, Mulder L, et al. Serum soluble interleukin-2 receptor measurement in patients with sarcoidosis: a clinical evaluation. Chest 2003;124:186-95.
51. Thi Hong Nguyen C, Kambe N, Kishimoto I, et al. Serum soluble interleukin-2 receptor level is more sensitive than angiotensin-converting enzyme or lysozyme for diagnosis of sarcoidosis and may be a marker of multiple organ involvement. J Dermatol 2017;44:789-797.
52. Petereit HF, Reske D, Tumani H, et al. Soluble CSF interleukin 2 receptor as indicator of neurosarcoidosis. J Neurol 2010;257:1855-63.
53. Ueda-Hayakawa I, Tanimura H, Osawa M, et al. Elevated serum BAFF levels in patients with sarcoidosis: association with disease activity. Rheumatology (Oxford) 2013;52:1658-66.
54. Tanimura H, Mizuno K, Okamoto H. Serum levels of soluble CD163 as a specific marker of macrophage/monocyte activity in sarcoidosis patients. Sarcoidosis Vasc Diffuse Lung Dis 2015;32:99-105.
55. Shamaei M, Mortaz E, Pourabdollah M, et al. Evidence for M2 macrophages in granulomas from pulmonary sarcoidosis: a new aspect of macrophage heterogeneity. Hum Immunol 2017.
56. Schreiber K, Nocturne G, Cornec D, et al. Lymphocytes as Biomarkers of Therapeutic Response in Rheumatic Autoimmune Diseases, Is It a Realistic Goal? Clin Rev Allergy Immunol 2017;53:277-290.
57. Judson MA, Baughman RP, Costabel U, et al. Efficacy of infliximab in extrapulmonary sarcoidosis: results from a randomised trial. Eur Respir J 2008;31:1189-96.
58. Chapelon-Abric C, Saadoun D, Biard L, et al. Long-term outcome of infliximab in severe chronic and refractory systemic sarcoidosis: a report of 16 cases. Clin Exp Rheumatol 2015;33:509-15.

59. Russell E, Luk F, Manocha S, et al. Long term follow-up of infliximab efficacy in pulmonary and extra-pulmonary sarcoidosis refractory to conventional therapy. Semin Arthritis Rheum 2013;43:119-24.
60. Vorselaars AD, van Moorsel CH, Zanen P, et al. ACE and sIL-2R correlate with lung function improvement in sarcoidosis during methotrexate therapy. Respir Med 2015;109:279-85.
61. Louis E, Mary JY, Vernier-Massouille G, et al. Maintenance of remission among patients with Crohn's disease on antimetabolite therapy after infliximab therapy is stopped. Gastroenterology 2012;142:63-70 e5; quiz e31.
62. Park W, Hrycaj P, Jeka S, et al. A randomised, double-blind, multicentre, parallel-group, prospective study comparing the pharmacokinetics, safety, and efficacy of CT-P13 and innovator infliximab in patients with ankylosing spondylitis: the PLANETAS study. Ann Rheum Dis 2013;72:1605-12.
63. Jung YS, Park DI, Kim YH, et al. Efficacy and safety of CT-P13, a biosimilar of infliximab, in patients with inflammatory bowel disease: A retrospective multicenter study. J Gastroenterol Hepatol 2015;30:1705-12.
64. Yoo DH, Hrycaj P, Miranda P, et al. A randomised, double-blind, parallel-group study to demonstrate equivalence in efficacy and safety of CT-P13 compared with innovator infliximab when coadministered with methotrexate in patients with active rheumatoid arthritis: the PLANETRA study. Ann Rheum Dis 2013;72:1613-20.
65. Schimmelpennink MC, Vorselaars ADM, van Beek FT, et al. Efficacy and safety of infliximab biosimilar Inflectra((R)) in severe sarcoidosis. Respir Med 2018;138S:S7-S13.
66. Lamberts SW, van der Lely AJ, de Herder WW, et al. Octreotide. N Engl J Med 1996;334:246-54.
67. Chadha MK, Lombardo J, Mashtare T, et al. High-dose octreotide acetate for management of gastroenteropancreatic neuroendocrine tumors. Anticancer Res 2009;29:4127-30.
68. Lichtenauer-Kaligis EG, Dalm VA, Oomen SP, et al. Differential expression of somatostatin receptor subtypes in human peripheral blood mononuclear cell subsets. Eur J Endocrinol 2004;150:565-77.
69. ten Bokum AM, Hofland LJ, de Jong G, et al. Immunohistochemical localization of somatostatin receptor sst2A in sarcoid granulomas. Eur J Clin Invest 1999;29:630-6.
70. Kwekkeboom DJ, Krenning EP. Somatostatin receptor imaging. Semin Nucl Med 2002;32:84-91.
71. Kwekkeboom DJ, Krenning EP, Kho GS, et al. Somatostatin receptor imaging in patients with sarcoidosis. Eur J Nucl Med 1998;25:1284-92.
72. Judson MA, Baughman RP, Costabel U, et al. Safety and efficacy of ustekinumab or golimumab in patients with chronic sarcoidosis. Eur Respir J 2014;44:1296-307.
73. Baughman RP, Lower EE, Bradley DA, et al. Etanercept for refractory ocular sarcoidosis: results of a double-blind randomized trial. Chest 2005;128:1062-47.
74. Liu C, Xia X, Wu W, et al. Anti-tumour necrosis factor therapy enhances mucosal healing through down-regulation of interleukin-21 expression and T helper type 17 cell infiltration in Crohn's disease. Clin Exp Immunol 2013;173:102-11.
75. Ignatenko S, Skrumsager BK, Mouritzen U. Safety, PK, and PD of recombinant anti-interleukin-21 monoclonal antibody in a first-in-human trial. Int J Clin Pharmacol Ther 2016;54:243-52.
76. Kotlarz D, Zietara N, Uzel G, et al. Loss-of-function mutations in the IL-21 receptor gene cause a primary immunodeficiency syndrome. J Exp Med 2013;210:433-43.
77. Salzer E, Kansu A, Sic H, et al. Early-onset inflammatory bowel disease and common variable immunodeficiency-like disease caused by IL-21 deficiency. J Allergy Clin Immunol 2014;133:1651-9 e12.

78. Butte MJ, Keir ME, Phamduy TB, et al. Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses. Immunity 2007;27:111-22.
79. Braun NA, Celada LJ, Herazo-Maya JD, et al. Blockade of the programmed death-1 pathway restores sarcoidosis CD4(+) T-cell proliferative capacity. Am J Respir Crit Care Med 2014;190:560-71.
80. Philips GK, Atkins M. Therapeutic uses of anti-PD-1 and anti-PD-L1 antibodies. Int Immunol 2015;27:39-46.
81. Cousin S, Toulmonde M, Kind M, et al. Pulmonary sarcoidosis induced by the anti-PD1 monoclonal antibody pembrolizumab. Ann Oncol 2016.
82. Grimbacher B, Hutloff A, Schlesier M, et al. Homozygous loss of ICOS is associated with adult-onset common variable immunodeficiency. Nat Immunol 2003;4:261-8.
83. Franke A, McGovern DP, Barrett JC, et al. Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. Nat Genet 2010;42:1118-25.
84. Sakthivel P, Grunewald J, Eklund A, et al. Pulmonary sarcoidosis is associated with high-level inducible co-stimulator (ICOS) expression on lung regulatory T cells--possible implications for the ICOS/ICOS-ligand axis in disease course and resolution. Clin Exp Immunol 2016;183:294-306.
85. Sullivan BA, Tsuji W, Kivitz A, et al. Inducible T-cell co-stimulator ligand (ICOSL) blockade leads to selective inhibition of anti-KLH IgG responses in subjects with systemic lupus erythematosus. Lupus Sci Med 2016;3:e000146.
86. Vincent FB, Morand EF, Schneider P, et al. The BAFF/APRIL system in SLE pathogenesis. Nat Rev Rheumatol 2014;10:365-73.
87. Mackay F, Woodcock SA, Lawton P, et al. Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations. J Exp Med 1999;190:1697-710.
88. Barbosa RR, Silva SP, Silva SL, et al. Primary B-cell deficiencies reveal a link between human IL-17-producing CD4 T-cell homeostasis and B-cell differentiation. PLoS One 2011;6:e22848.
89. Zhang P, Liu X, Guo A, et al. B Cell-Activating Factor as a New Potential Marker in Inflammatory Bowel Disease. Dig Dis Sci 2016;61:2608-18.
90. Chang SK, Mihalcik SA, Jelinek DF. B lymphocyte stimulator regulates adaptive immune responses by directly promoting dendritic cell maturation. J Immunol 2008;180:7394-403.
91. Munari F, Fassan M, Capitani N, et al. Cytokine BAFF released by Helicobacter pylori-infected macrophages triggers the Th17 response in human chronic gastritis. J Immunol 2014;193:5584-94.


Flinders Street
9

CHAPTER 7

ENGLISH SUMMARY
NEDERLANDSE SAMENVATTING

ENGLISH SUMMARY

Granulomas are organized clusters of immune cells containing macrophages, T cells and B cells. Granulomas are most often formed upon infection, such as tuberculosis, in order to contain the infection. Granulomas can also be formed in chronic immune mediated diseases such as sarcoidosis and Crohn's diseases as a sign of chronic inflammation. Both diseases were initially regarded Th1 mediated disease, yet increasing evidence has brought the involvement of Th17 cells, Tregs and B cells to the attention. Sarcoidosis and Crohn's disease are diseases with very different clinical presentations with mainly gastrointestinal involvement in Crohn's disease and a more systemic involvement in sarcoidosis commonly involving the respiratory tract, lymph nodes, eyes and skin. Despite these different clinical entities, treatment options are quite similar including corticosteroids, disease modifying drugs, such as methotrexate and azathioprine and tumor necrosis factor (TNF) blockers such as infliximab.

In **Chapter 2**, we studied the involvement of B cells in Crohn's disease in intestinal tissue and peripheral blood in patients without systemic treatment and during infliximab therapy. Granulomatous tissue of patients with Crohn's disease contained large B-cell infiltrates. Circulating transitional B cells and $CD21^{low}$ cells were elevated while IgM memory B cells were reduced in patients without systemic therapy. Patients responding well to infliximab did have normal numbers of transitional and IgM memory cells. Furthermore IgM memory B cells showed reduced replication histories and somatic hypermutation (SHM) levels suggestive of impaired generation, while switched IgA and IgG memory carried increased SHM levels indicating chronic stimulation. Together, these results suggest an aberrant and chronic B-cell response, which can be partially restored by infliximab.

In **Chapter 3** the earlier described B-cell infiltrates around granulomas in Crohn's disease were further studied. Granulomas are the most distinctive histopathological feature in Crohn's disease, yet are only found in up to a third of all cases. We hypothesized that by staining for B cells in biopsies of patients with Crohn's disease, the prevalence of granulomas could be increased. Indeed, granuloma rate increased with a 1.8 fold from 20% to 36% of cases. When subtotal colectomy samples of inflammatory bowel disease (IBD) patients were studied, three out of 39 patients retrospectively supported a diagnosis of Crohn's disease instead of ulcerative colitis. Furthermore dense B-cell infiltrates in Crohn's disease were significantly related to the presence of granulomas. Accurate diagnostic differentiation between Crohn's disease and ulcerative colitis can improve treatment outcome after surgery, but can also be important as many new disease specific treatment agents become available. Therefore including CD20 staining in standard IBD diagnostics can potentially improve the diagnostic process as well as disease management.

Chapter 4 investigated different immunological markers including sIL-2R, BAFF, infliximab trough levels and T – and B-cell subsets in patients with sarcoidosis starting infliximab therapy. Mixed therapeutic outcomes are reported in literature and we aimed through analyzing these markers to establish markers that determine potential response. 9/11 (82%) of patients in this study showed an objective clinical response. sIL-2r levels declined after the first infusion, while IgG and BAFF levels did not chance. Patients with a good and lasting clinical response showed increasing numbers of IgM^+ memory B cells as did regulatory T cells, Th17 and Th17.1 cells. Patients who did not show a lasting response during this study with formation of anti-drug antibodies (ADA) showed a different phenotype at baseline with lower levels of sIL-2R and Tregs, with higher IgM-only B cells when compared to responders to infliximab. This chapter confirmed the potential of monitoring of lymphocytic subsets, infliximab trough levels, sIL-2R and ADA for therapy success in patients with sarcoidosis.

In **chapter 5**, we studied the efficacy of infliximab in patients with neurosarcoidosis, a less common but often severe manifestation of sarcoidosis. In this retrospective, multicenter study, 28 patients were included. Most patients had a severe clinical presentation before infliximab was started with parenchyma localization, pituitary gland involvement, peripheral nerve involvement and chronic meningitis. Infliximab resulted in a complete remission in six(21%) patients, improvement in fourteen (50%) patients, stable disease in seven (25%) and deterioration in one (4%) patient. Furthermore, in the majority of patients (68%) successful discontinuation or tapering of corticosteroids was achieved. Relapse occurred in 26% of the patients after discontinuation of infliximab, yet most patients showed a therapeutic response again after reintroduction of infliximab. This study concludes that infliximab is an effective and relatively safe therapeutic agent in patients with neurosarcoidosis leading to clinical improvement or even remission in most patients.

Chapter 6 discussed the general findings described in the previous chapters. This thesis provides more evidence that B cells are actively involved in granuloma formation and/or preservation, possibly trough B-T cell interaction. Our studies with infliximab suggest that infliximab is a therapeutic agent in patients with sarcoidosis including pulmonary and extra-pulmonary disease and neurosarcoidosis. Immunological monitoring has the potential to further optimize response rates in patients starting treatment with infliximab. Still, a substantial fraction of patients with chronic inflammatory diseases have refractory disease. The complexity of treating these disorders is reflected by numerous failed drug trials. Our results with chronic sarcoidosis patients treated with sandostatin are of no exception as no clinically significant effect was observed.

With this thesis, we provide new evidence for the involvement of B cells in the immunopathogenesis of granulomatous inflammatory diseases. This opens new targeted treatment options for these disorders, by either targeting B cells or through B-T cell interactions.

NEDERLANDSE SAMENVATTING

Granulomen zijn georganiseerde clusters van afweercellen met onder andere macrofagen, T cellen en B cellen. Granulomen worden vaak gevormd bij infecties zoals tuberculose, om verder verspreiding in het lichaam te voorkomen. Echter kunnen granulomen ook gevormd worden in chronische immuun-gemedieerde ziekten zoals sarcoïdose en de ziekte van Crohn, als teken van langdurige ontsteking. Beide ziekten werden initieel gezien als Th1 gemedieerde ziekten, maar nieuwe inzichten suggereren ook betrokkenheid van Th17, regulatoire T cellen en B cellen. Sarcoïdose en de ziekte van Crohn zijn beide verschillende soorten ziekten met verschillende klinische kenmerken. Zo heeft de ziekte van Crohn voornamelijk gastro-intestinale betrokkenheid terwijl bij sarcoïdose bijvoorbeeld de longen, lymfklieren, ogen en huid kunnen zijn aangedaan. Ondanks de verschillende ziektekenmerken zijn de behandelingen die gebruikt worden veelal hetzelfde. Bij beide ziekten worden corticosteroïden (bijvoorbeeld prednison), immuun modulerende medicijnen zoals azathioprine en methorexaat en tumor necrosis factor (TNF) blokkers zoals infliximab gebruikt.

In **Hoofdstuk 2** hebben wij de betrokkenheid van B cellen bij de ziekte van Crohn bestudeerd door darmbiopten en bloed te onderzoeken van patiënten zonder medicijnen en patiënten behandeld met infliximab. Granulomateus darmweefsel van patiënten met Crohn lieten grote B-cel infiltraten zien. In het bloed werden grote hoeveelheden transitionele en $CD21^{low}$ cellen gezien terwijl IgM geheugencellen verlaagd waren bij patiënten zonder medicatie. Patiënten die goed reageren op infliximab lieten wel normale IgM geheugencellen zien. Daarnaast zagen wij op moleculair niveau dat IgM geheugencellen minder celdelingen hebben doorgemaakt met ook minder somatische hypermutaties (SHM). Dit suggereert een defect in de aanmaak van deze geheugencellen. Dit in tegenstelling tot IgA en IgG geheugencellen welke juist meer SHM hadden wat een indicatie is voor chronische stimulatie van deze cellen. Samen laten deze resultaten zien dat er sprake is van een veranderde en chronische B-cel respons, welke gedeeltelijk genormaliseerd wordt door infliximab.

In **Hoofdstuk 3** bestuderen wij de eerder beschreven B-cel infiltraten rondom granulomen uit biopten van patiënten met de ziekte van Crohn. Van alle verschillen in histopathologie tussen Crohn en colitis ulcerosa (een andere inflammatoire darmziekte), is het granuloom het meest kenmerkende verschil tussen deze ziekten. Het probleem is dat granulomen slechts in ongeveer een derde van alle Crohn biopten wordt gevonden. Onze hypothese was dat door het kleuren voor B cellen in deze biopten, wij meer granulomen zouden vinden. Het aantal gevonden granulomen steeg inderdaad met 1.8 keer van 20% naar 36% . Wanneer chirurgisch verwijderde stukken darm van subtotale colectomie operaties werd onderzocht, waren er daarnaast nog 3 van de 39 patiënten die achteraf meer een diagnose Crohn in plaats van colitis ulcerosa hadden. Daarnaast vonden wij een relatie tussen de hoeveelheid B cellen in een biopt

en de kans op granulomen; hoe meer B cellen hoe groter de kans dat er granulomen te zien waren. Het is belangrijk om goed onderscheid te kunnen maken tussen de ziekte van Crohn en colitis ulcerosa omdat dit een verschillende uitkomst in chirurgische opties kan betekenen, maar ook gezien nieuwe ziekte specifieke medicijnen die de komende jaren op de markt zullen komen. Daarom wijst dit onderzoek uit dat door CD20 (B-cel kleuring) aan de standaard diagnostische kleuring toe te voegen, mogelijk de diagnostisering van inflammatoire darmziekten kan verbeteren en daarmee ook de uiteindelijke behandeling van de juiste ziekte.

Hoofdstuk 4 laat verschillende immunologische markers zien zoals sIL-2R, BAFF, infliximab dalspiegels en T- en B-cel subsets in patiënten met sarcoïdose die gaan starten met infliximab behandeling. In de literatuur worden goede en minder goede resultaten met dit middel beschreven. Door middel van deze studie probeerden wij door het bestuderen van verschillende markers de potentiële therapeutische respons te kunnen bepalen. 9/11 (82%) van de patiënten in deze studie liet een objectief goede klinische respons zien. sIL-2R waarden daalden al na het eerste infliximab infuus, terwijl IgG en BAFF waarden niet veranderden. Patiënten die langdurig goed reageerden op infliximab hadden stijgende IgM geheugencellen naast ook regulatoire T cellen, Th17 en Th17.1 cellen. Patiënten die niet langdurig reageerden op infliximab met de vorming van autoantistoffen tegen infliximab, lieten voor de studie al lagere sIL-2R waarden en regulatoire T cellen zien met juist hogere IgM geheugencellen. Doordat patiënten die goed of juist niet goed reageren op infliximab al voor start van behandeling andere immunologische patronen laten zien kan het nuttig zijn om lymfocyten subsets, infliximab dalspiegels en sIL-2R waarden te vervolgen.

In **Hoofdstuk 5** bestuderen wij de effectiviteit van infliximab bij patiënten met neurosarcoidose, een minder vaak voorkomende maar vaak ernstige vorm van sarcoïdose. 28 Patiënten vanuit het Erasmus MC in Rotterdam en AMC in Amsterdam, zijn beschreven in deze retrospectieve studie. De meeste patiënten hadden een ernstig klinisch beeld bij presentatie met hersenparenchym, hypofyse of perifere zenuwbetrokkenheid en chronische meningitis. Zes (21%) patiënten kwamen in complete remissie, veertien (50%) lieten verbetering zien, zeven (25%) hadden stabiele ziekte en bij één (4%) patiënt verslechterde de ziekte. Daarnaast kon in de meerderheid van de patiënten (68%), steroïden therapie worden afgebouwd of gestaakt. Wanneer infliximab werd gestaakt kwam de ziekte in 26% van de gevallen weer terug. Echter bij herstarten van infliximab reageerden patiënten vaak toch weer goed op de medicatie. Infliximab is een effectieve en relatief veilig vorm van medicatie bij patiënten met neurosarcoïdose wat leidt tot klinische verbetering of remissie bij de meeste patiënten. **Hoofdstuk 6** bevat de resultaten uit de voorgaande hoofdstukken. Dit proefschrift levert meer bewijs dat B cellen actief betrokken zijn in de granuloom vorming en/of behoud hiervan, mogelijk door B - T cel interactie. Onze studies met infliximab laten zien dat het een effectief geneesmiddel lijkt te zijn in zowel pulmonale als extrapulmonale ziekte

en neurosarcoidose. Monitoring met immunologische markers kan mogelijk gebruikt worden om het succespercentage van infliximab nog verder te verbeteren. Daarnaast blijft er een groep patiënten met chronische inflammatoire ziekten die niet reageert op de gangbare medicatie. Dat er meerdere medicijnstudies zijn gepubliceerd die geen verbetering van ziekte laten zien, illustreert de complexiteit van deze ziekten. Onze eigen studie waarin wij patiënten met chronische sarcoïdose behandeld hebben met sandostatine is daarin geen uitzondering; ook hier werd geen klinisch effect worden aangetoond.

Met dit proefschrift wordt nieuw bewijs geleverd voor de betrokkenheid van B cellen bij de immunopathogenese van granulomateuze inflammatoire ziekten. Dit biedt kansen voor nieuwe vormen van therapie; bijvoorbeeld door therapie gericht tegen B cellen of B - T cel interactie.


Flinders Street
9

DANKWOORD

Ineens is het zover, het dankwoord is het allerlaatste wat nog geschreven dient te worden in dit proefschrift. Velen hebben bijgedragen aan de totstandkoming van dit proefschrift; van begeleiding van supervisoren, hulp van vele collega's en het eindeloze begrip van familie en vrienden. Mijn dank is groot!

Beste Prof.dr. van Hagen, beste Martin, al in het begin van mijn studie Geneeskunde was ik onder de indruk van jouw geweldige enthousiasme en kennis van de Klinische Immunologie. Dank voor de begeleiding de afgelopen jaren, de vrijheid en het vertrouwen dat ik kreeg bij het opzetten van nieuwe studies, bezoeken aan het buitenland en de steun bij het afronden van dit proefschrift en het starten van mijn opleiding.

Beste dr. van Laar, beste Jan, jij hebt letterlijk aan de wieg van dit proefschrift gestaan door mij als tweedejaars Geneeskunde student te enthousiasmeren voor de Immunologie na een casusbespreking over systeemziekten. Jij werd mijn mentor en ik mocht meelopen op de poli, grote visites bijwonen en assisteren met onderzoek. Dit heeft uiteindelijk geleid tot mijn afstudeeronderzoek, oudste co-schap en het starten van mijn promotieonderzoek op jullie afdeling. Dank voor jouw niet aflatende steun, mentorschap en positivisme in al deze jaren.

Beste dr. van Zelm, beste Menno, jij kreeg er ineens een student bij in jouw lab die nog niet eens normaal kon pipetteren. Uiteindelijk bleek dat dit best aan te leren was en ben ik uitgegroeid tot volwaardig BCD lid. Jouw kritische blik en streven naar diepgang hebben dit proefschrift zeker naar een hoger niveau getild en van mij een beter onderzoeker gemaakt. Jouw vertrek naar Melbourne was niet altijd gemakkelijk voor de groep, maar heeft mij de unieke mogelijkheid geboden een half jaartje in jouw lab te mogen werken, waar ik je erg dankbaar voor ben. Het gaat jullie goed in Australië!

Prof.dr. Hofland, prof.dr. Baeten, prof.dr. Aerts, prof.dr. van der Woude, prof.dr. van Saase, prof.dr. van de Beek; hartelijk dank voor de tijd en moeite om mijn proefschrift kritisch door te nemen en jullie bereidheid plaats te nemen in de promotiecommissie.

Beste dr. Lam, beste King, initieel stond jij niet heel erg te kijken op mijn verzoek om granulomen te gaan zoeken in darmbiopten. Toen wij granulomen vonden, sputterde jij eerst nog dat het geluk was; uiteindelijk bleek jij de enthousiasteling die avonden darmweefsel ging zitten scoren op B-cel aantallen. Dankjewel voor je humor, je wijze raad en steun die mij door die stapels biopten hebben geholpen en je bereidheid in de commissie deel te nemen.

Hierbij wil ik alle coauteurs bedanken voor de geleverde bijdragen aan publicaties en dit proefschrift.

Beste Paul en Virgil dank voor jullie bijdrage aan het sandostatine manuscript. Daarnaast ben ik ook dankbaar voor de vele klinische lessen die ik van jullie heb geleerd in al die jaren en gezelligheid op congressen.

Beste Wim en Marco, dank voor de bijdrage aan de follow up sarcoïdose studie, maar daarnaast ook voor jullie kritische vragen tijdens meetings en de bereidheid van jullie groep om te helpen met het verwerken van samples als dat nodig was.

Beste dr. Ouwendijk, Corry, Lenny en Conny van het Ikazia Ziekenhuis, dank voor jullie hulp bij de inclusie van Crohn patiënten en de gezelligheid als ik bloed kwam ophalen.

Beste Mirjam, dank voor de steun en wijze raad wanneer ik dat zo nodig had. Veel succes met jouw nieuwe stap in Leiden.

Sarcoïdose is bij uitstek een ziekte die de expertise van meerdere specialismen behoeft. Dank ook aan dr. Wijsenbeek, drs. Miedema, drs. Siepman en de andere specialisten die hierbij betrokken zijn in het Erasmus MC. Speciale dank aan dr. Brouwer en Daan Fritz van het AMC; mooi dat wij onze krachten hebben gebundeld en data hebben samengevoegd in het neurosarcoidose infliximab manuscript.

Beste Judy, dankjewel voor jouw hulp bij alle grant-aanvragen en jouw hulp met het afronden van dit proefschrift.

Beste Lieke, dankjewel voor de kans die je mij hebt gegeven te assisteren bij jouw onderzoek en de begeleiding van mijn afstudeeronderzoek.

Lieve Christina en Britt, wat ben ik blij dat jullie mijn paranimfen willen zijn. Door dik en dun hebben jullie mij gesteund in al die jaren, hele therapeutische sessies zijn over onze app gegaan! Jullie promoties zijn de volgende!

Jorn, samen begonnen als student bij Menno op het lab, samen promotieonderzoek gedaan, samen naar Australië, allebei in opleiding tot internist en nu promoveren op dezelfde dag! Ik waardeer onze tijd in Australië heel erg en ben blij dat ik jou nog vaak zal tegenkomen in de komende jaren.

Lieve Naomi, toen ik bij jou op de werkkamer kwam was je niet heel blij…hoe is dat veranderd! Ik had mij geen betere roomy kunnen wensen met alle steun en gezelligheid; gelukkig ook nu we in twee verschillende werelddelen wonen. Ik ben zo trots op hoe goed jij het doet in New York!

Willem-Jan, wij begrepen elkaar als clinici tussen alle biologen. Wat een lol was het met jou, met als hoogtepunt onze gezamenlijke roadtrip in Australië met Mel en Anton. Ik ga jou nog vaak in consult vragen.

Ruud, toen jij bij Menno begon was jij bijna gepromoveerd en bijna kinderarts. Allebei delen wij een liefde voor systeemziekten en sarcasme en dat bleek de basis van onze vriendschap. Veel succes terug in Toronto!

Tim (vd Houwen) en Tim (Both); alle drie als student begonnen bij de Klinische Immunologie en nu in opleiding tot internist. Onze tripjes naar de Wetenschapsdagen in Antwerpen zijn onvergetelijk. Ook jullie ga ik nog vaak tegenkomen gelukkig.

Diana, jij liet mij zien dat je soms moest vechten om tot een afgerond proefschrift te komen, want onderzoek doen is lang niet altijd gemakkelijk of leuk. Op jou kon ik altijd terugvallen met flowcytometrie, staining of gating problemen. Of eigenlijk ieder ander probleem; bedankt!

Dear old-BCD group; Magda, Magda, Benjamin and Edwin; thank you for taking me in the group and helping me out all those years!

Aan alle (oud) promovendi en postdocs van de afdeling Immunologie; dank voor de input bij alle meetings en gezelligheid bij congressen en PhD uitjes. Special thanks to the roomies in the final stretch of my PhD: Dew, Jamie and Iris. Prayer, Chris and Rina thank you for your support, the laughter and fun we shared at conferences and nice dinners we had. Liza, jij bracht de Brabantse gezelligheid op de afdeling, waarvoor dank.

Dear people of the 'van Zelm group' and of the Dept. of Immunology and Pathology of Monash University in Melbourne. Thank you all for giving me the best experience in your lab with learning new things but also the joy and laughter, drinks and dinners we shared. It was the best! Special thanks to Pei for taking on our project and processing all those samples!

Beth, my best and only roommate, thank you for making my time in Melbourne even more memorable to me!

Beste dr. van Kasteren en stafleden Interne Geneeskunde van het ETZ in Tilburg. Dank voor jullie hulp en vertrouwen in mij bij mijn terugkeer naar de kliniek. Ik leer iedere dag weer van jullie.

Lieve assistenten Interne Geneeskunde van het Elisabeth; wat is het een feest om deel uit te mogen maken van dit hechte team. Bedankt voor alle hulp en flexibele opstelling rondom het afronden van dit proefschrift. Het is mooi om te zien dat wij er voor elkaar zijn als het nodig is, maar ook samen mooie feestjes kunnen bouwen.

Lieve Eline, Femke, Hanna en Nathalie; ik leerde jullie allemaal ruim elf jaar geleden kennen in de eerste weken van de studie Geneeskunde en wat ben ik blij dat ik jullie (plus partners en kinderen) nog steeds mijn vrienden mag noemen.

Beste Bert, Margriet, Martijn en Thymen Kerver, dank voor jullie steun en gastvrijheid in Rotterdam, we weten dat we bij jullie altijd welkom zijn!

Lieve Maarten en Josefa, thank you for years of support, dinners and fun trips. Josefa thank you so much for editing parts of this thesis. Maarten, jij bent meer dan alleen een (peet)oom voor mij, onze band is heel bijzonder.

Lieve opa en oma, wat ben ik blij dat jullie er bij zijn vandaag. Dankjewel dat jullie zo lang en goed voor Anton hebben gezorgd voor onze definitieve verhuizing naar Eindhoven.

Lieve pap en mam, dank voor het ongekende vertrouwen wat jullie altijd in mij hebben gehad, ook als ik dat zelf even niet zo zag. Als klein meisje herinner ik me dat jullie mij voorhielden dat ik alles kon worden wat ik wilde. En dat meenden jullie; ook al had papa mij stiekem graag op de boerderij gehouden. Het harde werken wat met de paplepel is ingegoten bleek ook de afgelopen jaren goed van pas te komen… Stefan en Mark, ik ben blij met twee nuchtere broers als jullie. Jullie zijn altijd welkom bij ons. Nu allebei de proefschriften zijn afgerond hopen wij wat vaker naar Zeeland te komen voor een goede barbecue.

Lieve Anton,
01100101 01101001 01101110 01100100 01100101 01101100 01101001 01101010
01101011 00100000 01101001 01110011 00100000 01100100 01100001 01100001
01110010 00100000 01101111 01101111 01101011 00100000 01101101 01101001
01101010 01101110 00100000 01110000 01110010 01101111 01100101 01100110
01110011 01100011 01101000 01110010 01101001 01100110 01110100 00101110
00100000 01001001 01101011 00100000 01101000 01100001 01100100 00100000
01101000 01100101 01110100 00100000 01111010 01101111 01101110 01100100
01100101 01110010 00100000 01101010 01101111 01110101 00100000 01101110
01101111 01101111 01101001 01110100 00100000 01100111 01100101 01101011
01110101 01101110 01100100 00101100 00100000 01101010 01101001 01101010
00100000 01100010 01100101 01101110 01110100 00100000 01101101 01101001
01101010 01101110 00100000 01100111 01110010 01101111 01101111 01110100
01110011 01110100 01100101 00100000 01110011 01110100 01100101 01110101
01101110 00100000 01100101 01101110 00100000 01110100 01101111 01100101
01110110 01100101 01110010 01101100 01100001 01100001 01110100 00101110
00100000 01000100 01100001 01101110 01101011 01101010 01100101 01110111
01100101 01101100 00100000 01100100 01100001 01110100 00100000 01101010
01101001 01101010 00100000 01100101 01110010 00100000 01100001 01101100
01110100 01101001 01101010 01100100 00100000 01110110 01101111 01101111
01110010 00100000 01101101 01100101 00100000 01100010 01100101 01101110
01110100 00101110


Flinders Street
9

ABOUT THE AUTHOR

Marieke Timmermans was born on November 19th in 1988 in Den Bosch, the Netherlands. She grew up on a farm in Aardenburg (Zeeland, The Netherlands) with her two brothers Stefan and Mark and their parents Bert and Mieke. She graduated secondary School 'Het Zwin College' in 2007 and started studying Medicine at the Erasmus University in Rotterdam.

Already in the second year of Medical School, Marieke became interested in the field of Clinical Immunology and started participating in outpatient and clinical patient meetings and assisted in research projects. In 2013, Marieke obtained her medical degree cum laude. She started working on her PhD project (promotor prof.dr. P.M. van Hagen) on the research she already started as a student; the involvement of B cells in granulomatous inflammatory diseases. She obtained several grants to finance the studies in this thesis and in 2016 she received a research award from the 'Sarcoidose Belangenvereniging Nederland', the Dutch sarcoidosis foundation. In 2016, Marieke took the opportunity to do a five-month working visit in the lab of her co-promotor dr.M.C. van Zelm at Monash University, Melbourne, Australia. Marieke returned back to clinical work in 2017 when she started working as an ANIOS in Internal Medicine at the ETZ hospital in Tilburg. In January 2018 Marieke was allowed to start her residency in Internal Medicine under the supervision of dr. M. van Kasteren; ETZ Tilburg and dr. A. Zandbergen; Erasmus MC Rotterdam.

In the beginning of Medical School, Marieke met her partner Anton Kerver and they supported each other through the writing of two dissertations and multiple residencies. They happily live together in Eindhoven.


Flinders Street
9

PHD PORTFOLIO

Name PhD Student:	Wilhelmina Maria Cornelia Timmermans
Erasmus MC Department:	Internal Medicine and Immunology
Research School:	Molecular Medicine
PhD period:	January 2014 – October 2018
Promotor:	Prof.dr. P.M. van Hagen
Co-promotores:	Dr. J.A.M. van Laar
	Dr. M.C. van Zelm

PHD TRAINING

IN-DEPTH COURSES

2013	Basiscursus Klinische Onderzoekers (BROK)
2014	Biomedical English Writing
2014	Advanced Immunology
2014	Molecular Medicine
2014	WASOG conference, Izmir, Turkey
2015	Research Integrity
2015	Flowcytometry
2016	Seminars and minisymposia Dept. Immunology and Pathology Monash University
2014 – 2017	Regular seminars dept. Internal Medicine/Clinical Immunology
2014 – 2017	Seminars and minisymposia dept. Immunology Erasmus MC

TEACHING

2013 – 2016	Lectures Winter-course master Infection and Immunity
2014 – 2015	Supervising research projects medical students
2015 – 2016	Clinical Immunology cases, bachelor medicine

PRESENTATIONS

2012 – 2016	Internal Medicine Science Days, Antwerp, Belgium; *yearly presentations*
2013	WASOG, Paris, France; *poster presentations*
2014	Molecular Medicine day, Rotterdam, The Netherlands; *poster presentation*
2014	NVVI Annual Meeting, Kaatsheuvel, The Netherlands; *poster presentation*
2015	'Sarcoïdose Belangenvereniging' meeting with patients, The Netherlands; *oral presentation*
2015	EWIMID Amsterdam, The Netherlands; *poster presentation*
2015	NVVI Annual Meeting, Noordwijkerhout, The Netherlands; *poster presentation*

AWARDS AND FUNDS

2013	Travelgrant Gerrit Jan Mulder Stichting WASOG Paris
2014	Novartis investigator initiated trial: 'Sandostatin treatment in sarcoidosis'
2015	Grant Coolsingel Foundation project: 'Op zoek naar het granuloom'
2015	Grant CCU Foundation (Dutch Digestive Foundation) project: 'Op zoek naar het granuloom'
2015	Research award Sarcoïdose Belangenvereniging Nederland
2015	Travelgrant Erasmus Trustfonds for research visit Monash University
2015	Travelgrant NVVI for research visit Monash University
2016	Travelgrant Catharina van Tussenbroekstichting for research visit Monash University


Flinders Street
9

LIST OF PUBLICATIONS

1. Blood B- and T-cell kinetics, SIL-2R, infliximab trough levels and ADA formation indicate therapeutic succes of Infliximab in patients with sarcoidosis.
   Wilhelmina MC Timmermans, Willem A Dik, Marco WJ Schreurs, Jelle R Miedema, Marlies S Wijsenbeek, P Martin van Hagen, Menno C van Zelm, Jan AM van Laar – *Submitted*

2. Improved detection of granulomas by staining for B-cells: implications for differential diagnosis between ulcerative colitis and Crohn's disease.
   Wilhelmina MC Timmermans, King H Lam , Folkert J van Kemenade, Paul LA van Daele, P Martin van Hagen, Jan AM van Laar, Menno C van Zelm – *Submitted*

3. Sandostatin therapy in patients with chronic sarcoidosis.
   Wilhelmina MC Timmermans, Jan AM van Laar, Virgil ASH Dalm, Boen L Kam, P Martin van Hagen, Paul LA van Daele – *Published Sarcoidosis vasculitis and diffuse lung diseases, 2017*

4. Immunopathogenesis of granulomas in chronic autoinflammatory diseases.
   Wilhelmina MC Timmermans, Jan AM van Laar, P. Martin van Hagen, Menno C. van Zelm – *Published Journal of Clinical and Translational Immunology, 2016*

5. B-cell dysregulation in Crohn's disease is partially restored with infliximab therapy.
   Wilhelmina MC Timmermans, Jan AM van Laar, Tim B van der Houwen, Lieke SJ Kamphuis, Sophinus J Bartol, Rob J Ouwendijk, P Martin van Hagen, Menno C van Zelm – *Published Plos One, 2016*

6. The effect on memory B-cells in patients with Behçet's disease treated with TNF-blockers.
   Tim B van der Houwen, P Martin van Hagen, **Wilhelmina MC Timmermans**, Sophinus J Bartol, Jasper H Kappen, Menno C van Zelm, Jan AM van Laar – *Published Rheumatology, 2016*

7. Somatostatin receptor scintigraphy in patients with sarcoidosis.
   Lieke S Kamphuis, P Martin van Hagen, Tom O Missotten, G Seerp Baarsma, Virgil ASH Dalm, Willem A Dik, **Wilhelmina MC Timmermans**, Jan AM van Laar and Dik J Kwekkeboom – *Published Clinical Nuclear Medicine , 2015*


Flinders Street
9
FLINDERS STREET
PT
PT
GRAFF