Action and Function of Neutrophils in Crohn`s Disease

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Colophon

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Action and Function of Neutrophils in Crohn`s Disease

De rol van fagocyten bij de ontwikkeling van de ziekte van Crohn

Thesis

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born in Chennai (Madras), India

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Eelke Toxopeus

Whatever happened, it happened well
Whatever is happening, it is happening well
Whatever will happen, it will also happen well
What of yours did you lose?
Why or for what are you crying?
What did you bring with you, for you to lose it?
What did you create, for it to be wasted or destroyed?
Whatever you took, it was taken from here.
Whatever you gave, it was given from here
Whatever is yours today, will belong to someone else tomorrow.
On another day, it will belong to yet another.
This change is the law of universe

The Bhagavad Gita

CONTENTS

Chapter 1

Introduction

Adapted from:

Role of defective autophagia and the intestinal flora in Crohn`s

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Abstract

The precise mechanisms underlying the development of Crohn's disease (CD) remain controversial, but sufficient data have been collected to suggest that an uncontrolled immune response within the intestinal mucosa leads to inflammation in a genetically susceptible host. Although lack of mucosal regulatory T cells causes colitis in humans and experimental rodents, patients with CD have more rather than less regulatory activity in the intestine, apparently excluding defects in tolerance as the cause of CD. Genome-wide association studies have identified many gene variants that confer susceptibility and which seem associated to diminished functioning of especially innate immunity. In apparent agreement, CD patients are impaired with respect innate immune responses and controlling bacterial flora in the intestine. Furthermore, severe genetic deficiencies in innate immunity, like e.g. lack of NADPH oxidase activity or diminished function of the Wiskott Aldrich syndrome protein are associated with colitis in mice and men, and are often mistakenly diagnosed as CD. Thus we favour the view that the primary defect in CD is a lack in innate immunity, causing second tier immunological defences to combat otherwise easily controlled bacterial breaches of the mucosal barrier.

(1) The Nature of inflammatory bowel disease (IBD)

The primary function of the intestinal tract is the absorption of nutrients and secretion of waste products, both of which take place at the gastrointestinal tract (GI) mucosal lining, which consists of a thin permeable epithelium directly exposed to the external environment, called the lumen. The intestinal tract can be considered the largest surface in humans that is constantly exposed to a variety of environmental antigens and pathogenic microbes, as well as housing a large community of commensal bacteria. Its function in food and water absorption necessitates that the major part of the intestine consists of a single layer of epithelial cells, ill-adapted to withstand the mechanical wear and tear associated with the passage of food. Despite the fact that the intestinal tract is protected to some degree by a mucosal layer, bacteria constantly pass the epithelial layer and the entire intestine is in a constant state of low-grade inflammation. Nevertheless, the regulatory mechanisms present usually limit this inflammation to a subclinical state. In IBD, however, deregulation of these mechanisms results in chronic episodes of gastrointestinal inflammation, interspaced by periods of remission.

(2) Clinical features:

The two major types of IBD are Crohn`s disease (CD) and Ulcerative colitis (UC). Despite the overlapping pathological and clinical characteristics of CD and UC, they also show several distinctive pathological features. CD can be distinguished from UC by clinical phenotypes with respect to location and nature of the inflammation. In about 50% of the patients, the terminal ileum is involved, although CD may affect the entire gastrointestinal tract from the mouth to the perianal area. In about 30% of the patients the disease is located in both the ileum and colon and in approximately 20% the disease is limited to the colon. Areas of inflammation typically reveal discontinuous transmural involvement that, depending upon their location and severity, often lead to development of complications such as microperforations and fistulas, abdominal abscesses or granulomas. UC, like CD, is a relapsing inflammatory disease, but in adults is characterized by the presence of continuous inflammation limited to the mucosal layers and occasionally the submucosa of the colon, although in pediatric patients the entire tract may be involved. Typically, in adult UC, the disease often involves the rectum and extends proximally, but remains restricted in the colon. Although CD and UC are two different diseases, both have overlapping symptoms, including abdominal pain, vomiting, diarrhea, fever, hematochezia and weight loss. At least 25% of IBD patients develop extra-intestinal manifestations of the disease, indicative of auto-immune inflammatory processes, such as primary sclerosing cholangitis, erythema nodosum, pyoderma gangrenosum, uvieits, episcleritis, pleuritis, mycocarditis, peripheral arthritis, extra-intestinal cancer[1] and ankylosing spondylitis[2-4]. To date, no definite diagnostic test for IBD exists. Currently, the initial diagnosis is made on the basis of patient history and physical examination along with objective findings from endoscopy, radiological, laboratory and histological studies. The appearance of the human colon as viewed by colonoscopy in normal condition, CD and UC is shown in Figure 1. The peak age of onset of IBD is between 15 and 30 years, although a second peak is seen between ages of 50-80. About 10% of the cases occur in individuals under

the age of 18. UC is slightly more common in males, whereas CD is marginally more frequent in females. In clinical practice, about half of the patients of a given cohort are in clinical remission, which means that they are free of symptoms, and includes those who effectively respond to medical or surgical intervention. Overall, IBD patients have a normal life expectancy[4].

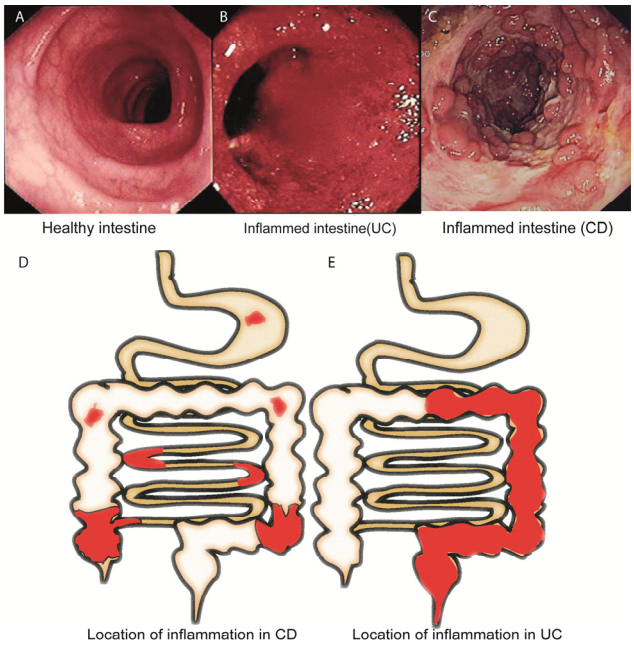

Figure 1: The appearance of human colon during colonoscopy (A) colon of a healthy individual; (B) colon of a UC patient with confluent superficial ulceration and spontaneous hemorrhage; (C) colon of a CD patient showing patch inflammation; (D) In CD, patchy inflammation may be located throughout the entire gastrointestinal tract, but is most commonly observed in the terminal ileum.; (E) continuous inflammation is restricted to the colon in UC patients.

(3) Epidemiology of IBD:

Epidemiological studies have shown that IBD has a combined prevalence of 300-400 cases per 100,000 people in northern climates of well-developed areas, such as North America and northern Europe and incidence rates ranging from 3-14 cases per 100,000 people. Although IBD occurs less frequently in ethnic or racial minority groups compared to Caucasians, this difference seems to be decreasing[5]. The prevalence and incidence is the lowest in southern climates and underdeveloped countries, such as South America, Southeast Asia and Africa[5], but increases fast in incidence in these regions as well, especially in Brazil and China. This variation in incidence rates significantly depends on geographic location and may be a result of environmental factors, such as industrialization, sanitation, hygiene and access to specialized health care[6]. The prevalence and incidence rates also differ in different racial (e.g. African Americans, Asians, Hispanics, Caucasians) and ethnic status (e.g. Jewish vs. non-

Jewish)[7, 8], implicating an important role for environmental factors as well as genetic influences.

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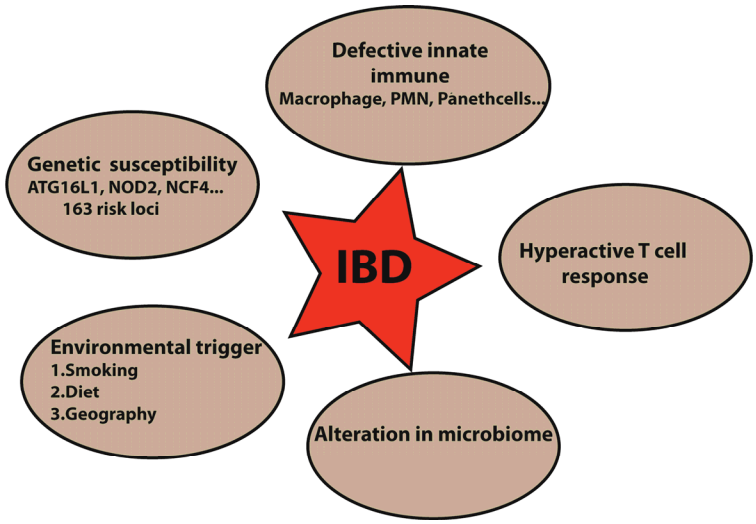

Figure 2: IBD is a multifactorial disorder. A defective innate and overactive adaptive immune response against the microbiome in a genetic susceptible host exposed to unfavorable environmental factors is the ideal situation for the development of IBD.

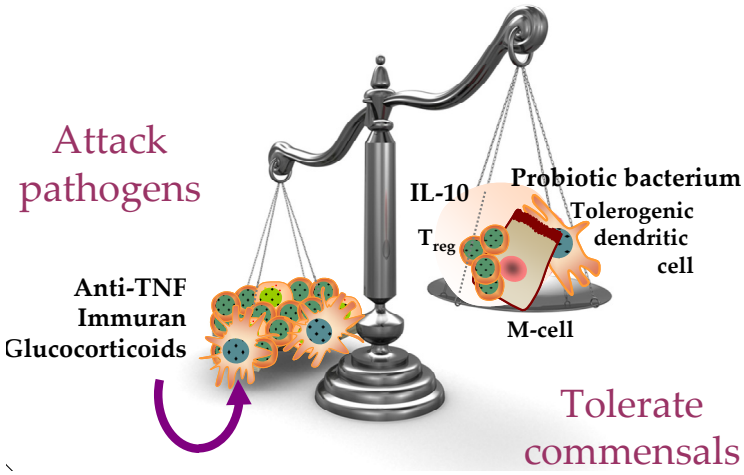

Figure 3: Classical model for Crohn's disease. When the balance between immunostimulatory and tolerogenic signals is disturbed, IBD may ensue and thus curing IBD would entail rectifying this misbalance.

(4.1) Role of reduced tolerance

The mucosal immune system enables us to fight pathogens while preventing responses against harmless substances/pathogens using ignorance or the induction of tolerance. As such, dysregulation of this immune system could encompass either the inability to fight pathogens or lead to autoimmunity. IBD is an example of this later immune dysfunction, where a loss of ignorance or tolerance towards the intestinal microbiotia leads to chronic inflammation in the intestine. Evidence for this comes from observations that rodents reconstituted with immune systems that lack regulatory capacity develop intestinal flora-dependent colitis in so called transfer colitis models[14a] and inhibition of regulatory T cell activity causes CD-like colitis in man[13, 14]. In addition, genetically abolishing signalling of the tolerogenic hormone IL-10 is a well established model for colitis[14a], whereas exogenous application of IL-10 using genetically modified bacteria shows promise in treating severe CD in the clinic (A phase I trial with transgenic bacteria expressing interleukin-10 in Crohn's disease[15]) all pointing to the idea that lack of tolerogenic capacity is the problem in this disease (Figure 3). However, patients with CD have supernormal levels of regulatory T cells, especially in the inflamed lesions [16] and thus although reduced tolerance can cause colitis, it is not the root cause of CD and alternative explanations are called for.

(4.2) Genetics of IBD: diminished functionality of the innate immune system causing susceptibility to CD?

In addition to the contribution of environmental factors in the pathogenesis of IBD, strong evidence from epidemiological studies that examined the occurrence of CD or UC within different familial aggregations, implicate the importance of genetic influences in IBD. Up to 5-10% of affected individuals have at least one first-degree family member with IBD with the relative risk to siblings ranging from 5-35% for CD and 10-15% for UC, suggesting that positive family history is more common in CD patients than in UC patients[17, 18]. This notion was confirmed in studies of twins and those persons having a twin with IBD had the highest risk of developing IBD in comparison to the general population[18, 19]. In monozygotic twins, the concordance rate for CD is reported to range between 40-60% compared to 4-12% in dizygotic twins, which is almost comparable to all siblings[17, 18, 20]. The concordance rate for UC is less frequent in monozygotic twins as well as in dizygotic twins and ranges from 6-17% and 0-5% respectively[21]. Collectively, these family data suggest a stronger effect of genetic factors in CD compared to UC and led to the notion that knowledge of the risk genes involved could provide important insights into the pathogenesis of IBD.

To identify the gene variants conferring increased life-time risk for contracting IBD, two statistical genetic approaches have been applied. Genome-wide linkage mapping strategies analyse a relative limited number (300-5000) of known genetic markers (e.g microsatellites, restriction fragment length polymorphism (RFLP) associated with a certain phenotype of the disease) to identify genomic regions of a chromosome shared between affected individuals as candidate disease loci. Although this technique was successful in identifying over ten shared chromosomal regions for disease risk[22-24], the usefulness of this approach in complex diseases such as IBD is limited, because of the involvement of multiple different genetic interacting risk

factors and the non-genetic risk factors. The identified candidate disease loci are often large and contain multiple genes, often without any apparent relation between the gene products in these regions and the actual disease. Nevertheless, using this approach three polymorphisms (or single nucleotide polymorphisms [SNPs]) in *NOD2* (nucleotide-binding oligomerization domain containing 2), previously known as *CARD15* (caspase activated recruitment domain protein 15), were identified as alleles associated with increased propensity of contracting CD and thus *Nod2* was the first example of an IBD susceptibility gene[25, 26] . The cytosolic NOD2 protein plays an important role in the innate immunity and is mainly expressed in epithelial cells and Paneth cells[17, 18], which are located at the base of the intestinal crypts, as well as antigen presenting cells (APCs), such as macrophages, monocytes and dendritic cells[26, 27]. It functions as an intracellular pattern recognition receptor (PRR) for invading pathogenic bacteria, including commensals residing in the lumen of the intestinal tract that have entered the mucosa. Its major ligand is *N*-acetyl muramyl dipeptide (MDP), a degradation product of peptidoglycan, the structural component of the cell wall in Gram-positive bacteria[28-30]. Binding of MDP leads to activation of the nuclear transcription factor NF-κB pathway[26] and mitogen-activated protein kinase (MAPK) pathway, resulting in pro-inflammatory mediators, such as TNF-α, IL-1β and IL6[31]. Recent studies have revealed an impaired mucosal clearance of bacteria in *NOD2*-deficient mice[32], implicating that NOD2 may play a central role in mucosal immunity. Interestingly, Nod2 alleles associated with CD displayed reduced rather than enhanced capacity to activate the pro-inflammatory transcription factor NF-kB[26] . This finding, together with the observation that patients with Crohn's disease are often defective in functionality of their innate immune system[33], led to the suggestion that CD should be considered as an (innate) immune deficiency[34]. Furthermore, *in vitro* experiments showed that monocytes isolated from CD patients had lower phagocytic activity towards *Candida albicans* than those obtained from healthy individuals[35]. Moreover, both in humans as well as in animals, genetic defects that provoke reduced innate immunity, e.g. deficiency of NADPH oxidase or Wiskott-Aldrich syndrome, cause CD-like colitis. Taken together, the body of contemporary biomedical literature strongly supports the concept that monocyte dysfunction is to be associated with the pathogenesis of CD-like and other autoimmunity [36](Figure 4). Support from this notion also comes from analysis of other risk genes.

(4.3) Innate immunity and autophagy

Recent advances in high-throughput genotyping techniques and increased knowledge about the HapMap Project enabled researchers to perform genome-wide association studies (GWAS) for several complex diseases, with CD leading the way. In these hypothesis-free methods of genome scanning, up to 500,000-1000,000 SNPs across the human genome are examined in both individuals with the disease and in healthy controls. The frequencies of these genetic variants found are statistically compared between the two groups to identify any association with the SNP and disease. In comparison to the genome-wide linkage and association studies described above, which are restricted to study a relative small number of well-phenotyped patients with a limited number of genetic variants in a few selected genes of suspected involvement

in the disease pathogenesis and the lack of power to identify genes with a weak effect, GWAS make use of moderately sized cohorts, thereby increasing the homogeneity within the studied population and significantly reducing the number of false positives[37-39]. The GWAS carried out so far, have led to an increased number of known genetic risk factors and these discoveries reveal novel insight regarding to pathways or mechanisms involved in the disease pathogenesis. In 2009 the number of identified loci conferring susceptibility to CD and UC development were 30 and 18[40], respectively. By the end of 2012, owing to the latest large scale GWAS meta analyses, this number had reached 163[41]. Together, these SNPs account for approximately 25% of the genetic susceptibility to CD, indicating that more genetic components remain to be identified. More than half of these loci are shared between CD and UC, suggesting a shared pathogenesis, but also pointing towards disease specific genetic contributions[41]. Most of the gene variants provide support, broadly speaking, for the concept that reduced function of the innate immune system contributes to the susceptibility to CD.

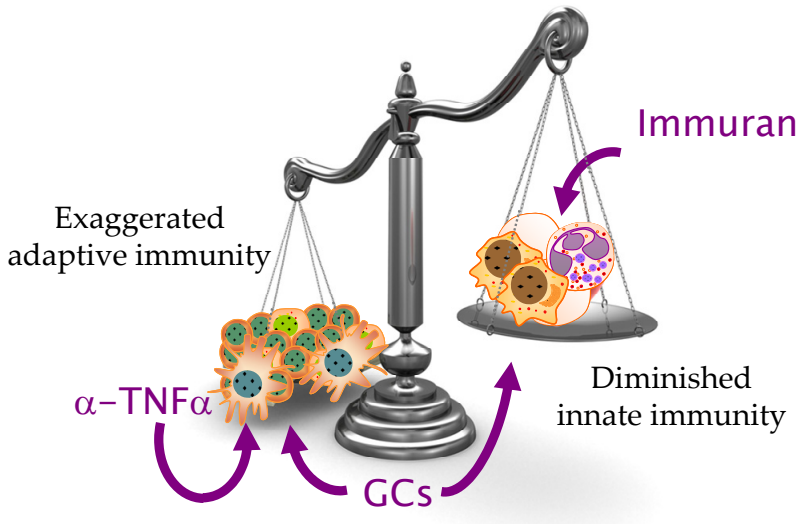

Figure 4: New model for Crohn's disease. Crohn's disease originates from reduced innate immunity and as a consequence a wrong balance between the innate and adaptive branches of host defense. Therapy rectifies this balance.

Remarkable within these, however, was the detection of the association with CD in multiple GWAS of two genes involved in autophagia, *ATG16L1* (autophagy-related 16-like 1) and *IRGM* (immunity-related GTPase)[40, 42-44]. Autophagy is a process by which cells encapsulate cytosolic debris, invaded pathogens, or old cellular organelles

destined for degradation and fuse these with the lysosomal apparatus[43a]. Defined by the marker rs2241880, a nonsynonymous amino acid change (threonine to alanine) at position 300 was found that carried all the disease risk for the *ATG16L1* locus and has been replicated in several independent cohorts[40, 43-47]. Several groups have provided evidence that this genetic association is highly associated with ileal CD[42, 44, 48-50]. Interestingly, this SNP resides in an evolutionary conserved domain of the ATG16L1 protein, located in exon 9 and translated into all known splice variants of *ATG16L1*[51]. This mutation seems to have a role in the protein stability and its interaction with other member proteins of the autophagic machinery[52]. ATG16L1 appears to be broadly expressed in intestinal epithelial cells, lymphocytes and macrophages[42, 44]. Although downregulation in *ATG16L1* mRNA expression was observed in colonic biopsies of CD patients[53], no significant differences in the levels of protein expression have been observed in intestinal tissue of CD patients versus healthy controls[42] and the expression of ATG16L1 was independent of the amino acid substitution T300A[51] . It is thus reasonable to assume that the susceptibility to Crohn's disease conferred by change of residue 300 in *ATG16L1* is consequence of altered function of the protein.
A variant for a second autophagy-related gene, *IRGM*, was detected for producing CD susceptibility in a WTCCC (Wellcome Trust Case Control Consortium) study[43]. In contrast to *ATG16L1*, no causative mutations associated with CD were detected in the coding region of IRGM, but a strong non-coding SNP (rs13361189) was found to be in perfect linkage disequilibrium with a 20-kb deletion polymorphism immediately upstream of the gene[43, 44, 54], implicating the involvement of regulatory sequences that control protein expression or post-transcriptional events, such as splicing. Compared to unaffected individuals of the reference population, the deletion allele showed an increased frequency in IBD patients, including association to CD and UC[54]. Studies have shown the importance of IRGM in eliminating intracellular pathogens, such as *Mycobacterium tuberculosis* by INF-γ mediated autophagy[55, 56] and several knockdown and overexpression experiments with IRGM show an altered efficiency of anti-bacterial autophagy. IRGM is differentially expressed in several tissues, including colon, small intestine, macrophages and monocytes[43], although these expression levels are low and it is difficult to detect endogenous IRGM[57].

Impaired innate immunity predisposes to CD, suggesting that the polymorphisms in autophagia-related genes that confer increased susceptibility to CD may somehow be related to changes in innate immune functionality. The ability of cells to maintain a constant internal environment is dependent on the balance between their synthesis and degradation processes. In eukaryotes, the evolutionary conserved autophagy response is used to keep this homeostasis. Autophagy is a process that enables cells to recycle unnecessary or damaged components in a highly regulated fashion. During nutrient-rich conditions, autophagy is simply activated to degrade long-lived or misfolded proteins and to dispose damaged cytosolic organelles, such as leaky mitochondria, thus preventing unwanted apoptosis and even potentially toxic aggregates[58]. In response to cellular stress events, such as nutrient deficiency, autophagy becomes strongly induced in order to supply the cell of nutrients (e.g. amino acids and energy (ATP)) through catabolism of the cells own constituents[59, 60]. In addition to these physiological described functions of autophagy, maintaining

homeostatic and cell survival, it also plays a role in host defence responses by promoting elimination of intracellular pathogens, including viruses, parasites and bacteria in a more selective manner, a process also referred to xenophagy. This defence mechanism has been widely studied in several pathological processes in eukaryotic organisms[60, 61] and is now implicated in a wide range of human diseases, including autoimmunity and inflammatory disorders with a direct relevance to the regulation of innate immune responses[62-64].

Specific evidence linking CD-susceptibility polymorphisms in autophagic genes to diminished autoimmunity comes from experiments with siRNA's directed at ATG16L1, which prevented autophagy of *Salmonella typhimurium* in the HeLa cell-line, suggesting that diminished function of ATG16L1 can indeed reduce innate immunity[44]. Insight into the possible functioning of ATG16L1 was gained from experiments in which mice were engineered to express a hypomorphic allele of Atg16L1. Paneth cells, which produce large amounts of protein and are hence susceptible to autophagic processes, showed an aberrant phenotype in these animals[65]. Paneth cells control the small intestine bacterial flora through the production of defensins and thus impaired Paneth cell function may be considered a specialized innate immunodeficiency. Although extrapolating these observations from a highly artificial murine model to the human situation is not completely straight-forward, Cadwell et al. do present evidence that patients homozygous for the ATG16L1 risk allele have a Paneth cell phenotype, and the ileal localization of disease is linked to this allele (the colon does not contain Paneth cells). Indeed, CD is characterized by a specific decrease in ileal Paneth cell alpha-defensins. Hence, autophagia-related CD might constitute a specialized form of reduced innate immunity-dependent IBD.

(5) Role of granulocyte functions in IBD

(5.1) Deficient antimicrobial response against the pathogens

Among the most important mediators of innate immunity are polymorphonuclear granulocytes (PMN); professional phagocytes which mount the acute inflammatory response, acting as the first line of defence against invading pathogens[70, 71]. The importance of the first line of defence is underscored by the fact that patients with decreased numbers of circulating PMN have a high mortality rate due to recurrent life-threatening bacterial infections[72]. In order to perform their bactericidal activity, PMN have to perform a number of steps (Figure 5): (1) adhesion to and rolling along endothelial lining of blood vessels, (2), diapedesis through the endothelial layer, (3) migration towards the site of inflammation, (4) degranulation and finally (5) phagocytosis of bacteria in conjunction with production of bactericidal reactive oxygen species (ROS). ROS production is mediated by the nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase complex, which consists of 6 proteins. Two membrane-bound proteins $p22^{phox}$ and $p91^{phox}$ (encoded by *CYBA* and *CYBB*, respectively) together form flavocytochrome b558. Upon stimulation of PMN, the four cytosolic proteins $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and p21Rac2 (encoded by *NCF1*, *NCF2*, *NCF4* and *Rac2*) translocate to the plasma membrane, associate with flavocytochrome b558 to form the active NADPH oxidase, allowing the transfer of electrons and the production of ROS (Figure 6). Defects in cytokine-mediated activation of signal transduction

pathways involved in PMN function, including the phosphatidylinositol 3-OH kinase-protein kinase B (PKB) and extracellular signal regulated kinase (ERK1/2) pathways, have been shown to contribute to PMN associated diseases[73]. Many immunodeficiencies that arise as a result of impaired granulocyte function present with a CD-like phenotype. For instance, in patients suffering from chronic granulomatous disease (CGD), mutations in the NADPH oxidase complex genes *NCF1*, *NCF2*, and sporadically *NCF4*, result in impaired PMN respiratory burst. Approximately 50% of CGD patients present with IBD-like symptoms, demonstrating the importance for proper ROS homeostasis in the maintenance of a healthy mucosa. This notion is supported by recent experiments in mice, showing that knock down of *NCF4* results in PMN-dependent colitis. Other bactericidal functions have also been implicated in CD development. PMN from patients suffering from glycogen storage disease type-1b (GSD-1b) show impaired ROS production, phagocytosis, migration and calcium mobilization[74]. Again, up to 77% of GSD-1b patients develop IBD. Gastrointestinal involvement has also been observed in Wiskott Aldrich Syndrome, in which loss of WAS protein results in impaired cell migration, and Leukocyte adherence syndrome-1 (LAD-1), which is characterised by elevated PMN blood counts, reduced PMN migration, phagocytosis and bacterial killing[75]. All in all, these observations support a role for innate immune cell dysfunction in CD, and strongly suggest that intrinsic defects in PMN function contribute to this disease.

Recently, a hypothetical-driven search for novel mutations in specific gene classes resulted in the identification of a rare variant of NADPH-oxidase gene *NCF2* in very early-onset IBD patients[76, 77]. This mutation precluded the organisation of the NADPH oxidase complex by hampering the interaction of $p67^{phox}$ with the GTPase p21Rac2. Interestingly, GWAS analyses have also identified IBD-risk conferring SNPs in Rac2 itself, as well as in a third member of the NADPH oxidase, NCF4. Our own studies indicate that granulocytes from CD patients carrying this NCF4 risk allele have a reduced production of reactive oxygen species. Thus, CD genetic risk factors may contribute to CD pathology by altering the innate immune response. Another example of this is presented by NOD2, which is expressed in granulocytes, and depletion of which has been shown to result in an impaired ability to reduce intracellular bacterial burden[78]. Thus, defective innate immunity may result in enhanced persistence of bacterial load, and may help explain why commensal microbiota in CD patients is altered and contributes to disease. Interestingly, as mentioned above, many of the identified CD risks SNPs are found in genes that are involved in innate immune defence, and their specific effects await elucidation.

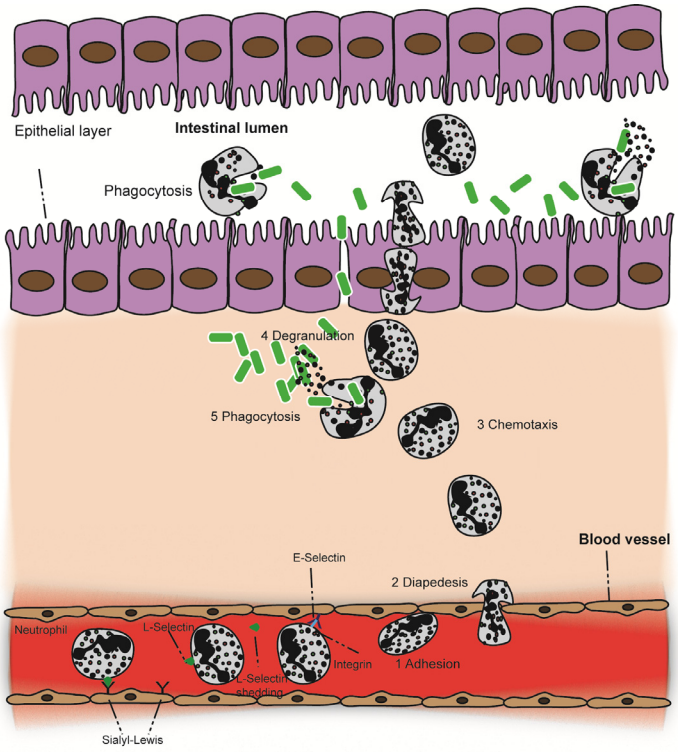

Figure 5: PMN migration and killing of pathogens at the inflamed site. In order to perform their bactericidal activity, PMN have to go through a number of steps (1) adhesion to and rolling along endothelial lining of blood vessels; (2) diapedesis through the endothelial layer; (3) chemotaxis towards the site of inflammation; (4) degranulation, releasing microbial enzymes and ROS, and (5) phagocytosis of bacteria and intracellular ROS production.

(5.2) PMN contribution in immune cell recruitment and activation in the gut

During the initial phase of intestinal inflammation, resident/surveillance monocytes sense the pathogens and release factors responsible for the recruitment of PMN[79, 80]. Once the PMN reach the site of infection or inflammatory site, they selectively release monocyte chemoattractants, such as Cathepsin G, CAP18 (Cathelicidin) and azurocidin, thus engaging a positive feedback loop[80]. This inflammatory response ensures the clearance of pathogens. In CD, it has been suggested that monocytes/macrophages are by nature deficient in synthesizing proinflammatory cytokines, which may limit the migration of PMN immigration to the site of inflammation[81]. Nevertheless, abundant numbers of PMN are commonly observed at the inflamed mucosa. PMN secrete significant levels of pro-inflammatory cytokines (TNFα, IL-1α, IL-1β, IL-6, IL-7, IL-9, IL-16, IL-18, MIF) and anti-inflammatory cytokines (IL-10, TGFβ, IL-1RA, IL-4) during inflammation[82-85]. Recently, it has been reported that PMN are capable of producing IL-17[86], which has been widely associated with pathological intestinal inflammation[87]. This suggests that IL-17 production by intestinal PMN may have a potential role in IBD,

an interesting notion which requires further investigation. PMN also produce a wide range of CXC- chemokines (CXCL1, CXCL2, CXCL3, CXCL4, CXCL5, CXCL6, CXCL8, CXCL9, CXCL10, CXCL11)[88] and CC-chemokines (CCL2, CCL3, CCL4, CCL17, CCL18, CCL19, CCL20, CCL22)[88] which are involved in the recruitment of immune cells (CD4 T cells, CD8 T cells, Th17, Tregs) to the inflamed site (Figure 7). In addition, PMN are capable of producing matrix metalloproteases (MMPs) which can either inactivate (MMP9 can inactivate growth-related oncogene α and platelet factor 4), enhance the potency of (CXCL1 & CXCL8[89]) or activate chemokines (MMP-8 cleaves CXCL5 and CXCL8[90]).

Although the inflamed mucosa of IBD patients show an influx of granulocytes and activated monocytes, the granuloma formation often observed in CD suggests a defective clearance of bacteria through this first wave of innate immunity. In turn, the restricted bacterial clearance by PMN may result in hyper-activation of dendritic cells (DCs)[14, 15] by overloading of bacterial antigens, which will be processed and presented to $CD4^+$ helper cells. Depending on the cytokines produced by DCs, naive T cells will be directed towards either a Th1 or Th2 phenotype. Based on the mucosal cytokine expression patterns in IBD, it is generally assumed that CD presents as a Th1 mediated immune disease (high levels of IL-12, INFɣ, TNFα and IL-2, whereas UC holds typical Th2 characteristics (high levels of IL-5, IL-10 but not IL-4) [18-20].
Through substantial experimental and clinical evidence it is becoming increasingly clear that defective innate immune system followed by uncontrolled T-lymphocyte activation could be a pathogenic mechanism of IBD[13, 91, 92]. As such, PMN can potently affect inflammatory conditions through the secretion or modification of cytokines and chemokines that have important roles in the recruitment of other immune cells in IBD.

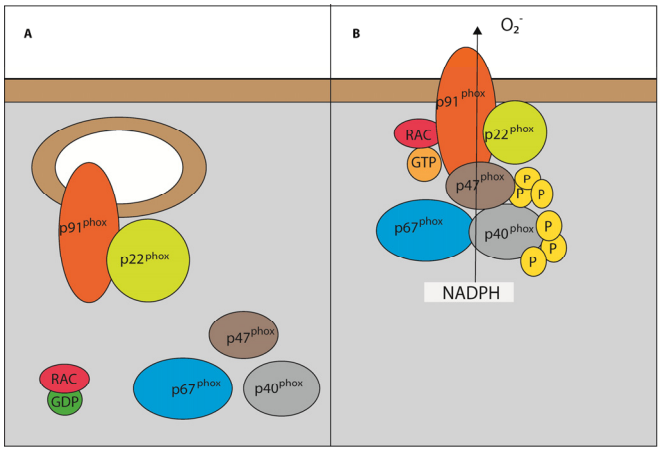

Figure 6: Recruitment of NADPH oxidase complex and reactive oxygen species production. (A) The NADPH oxidase consists of membrane bound ($p91^{phox}$, $p22^{phox}$) and cytosolic components ($p67^{phox}$, $p40^{phox}$, $p47^{phox}$ and the GTPase Rac2). (B) After stimulation of PMH, $p67^{phox}$, $p40^{phox}$ and $p47^{phox}$ become phosphorylated, Rac2 becomes activated, and all assemble at the plasma membrane to associate with the membrane component to form an active NADPH oxidase complex.

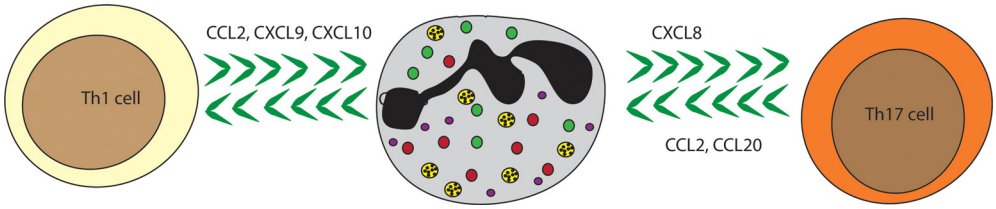

Figure 7: Interplay between PMN and T cells. PMN secrete chemokines (CCL2, CXCL9, CXCL10) which favor the recruitment of TH1 cells towards the site of inflammation, and CCL2 and CCL20 which enable the recruitment of TH17 cells.

(5.3) PMN as antigen presenting cells (APCs) during inflammatory situation like IBD

Aside from a role in T-cell recruitment, PMN may influence the adaptive immune response through a second mechanism. Interestingly, these professional phagocytes have been shown to be able to acquire a dendritic cell like phenotype, in that they can start to express MHC-II molecules and co-stimulatory molecules. It is as yet unclear whether loading of MHC-II with antigen-peptide occurs; however these re-differentiated cells nevertheless seem capable of activating T-cells. This response has been observed *in vivo* in disease types that have similarities to CD, such as rheumatoid arthritis[93]. In addition, PMN isolated from the inflamed colon in adoptive transfer mouse models were reported to express MHC-II and CD86[94]. The concept of PMN stimulating T-cell responses by acting as APC, thereby contributing to the known T-cell over-activation in IBD mucosa, is an interesting novel concept, investigation of which is urgently called for.

Aim and thesis outline

Mucosal inflammation in CD patients is thought to develop from a limited innate immune response and an overactive adaptive immunity to the commensal luminal bacteria. However, the role of phagocytes in CD development is not properly investigated. In this thesis, we discuss the role of PMN in the development of Crohn`s disease, linking their function to the genetic phenotype of patients.
The importance of choosing the ideal method for phagocyte (PMN and monocytes) isolation and to what extent different isolation methods can influence phagocyte functions are discussed in **chapter II**.

In **chapter III** various PMN functions such as migration, bacterial uptake, bacterial killing, reactive oxygen species production, apoptosis and survival are investigated in CD patients in remission. We show that PMN from CD patients have an inherent defect in some of their functions, demonstrating that impaired innate immunity as a result of aberrant PMN activity may indeed contribute to CD.
Numerous SNPs are predicted to be associated with an increased risk to develop CD. However, the relative contribution of these SNPs to disease development, in terms of proof showing their effect on any given cellular function, is lacking for most of these. One of the genetic risk factors identified for CD is an intronic SNP in the gene *NCF4*, which encodes for $p40^{phox}$, an essential NADPH oxidase protein. In **chapter IV** we show that PMN from patients bearing the NCF4 risk allele are impaired in their capacity to produce ROS.

As the SNP in NCF4 is intronic, and therefore is less likely to affect $p40^{phox}$ protein function, we wondered how the defect in ROS production in risk-allele carrying patients may be explained. In **chapter V** we show that impaired ROS production in PMN from NCF4 risk-allele patients is restricted to cells that are primed with the pro-inflammatory cytokine granulocyte macrophage colony stimulating factor (GMCSF). The receptor for GMCSF consists of a ligand-binding α-subunit, and a signal-transducing β-subunit. Interestingly, the gene encoding the β-subunit, *CSF2RB*, is located immediately downstream of *NCF4*. In this chapter, we show that the NCF4 risk allele confers a CSF2RB signalling defects in CD patients carrying this SNP.

In severe inflammatory conditions, PMN at the site of inflammation can express MHC II and co-receptors that are normally found on antigen presenting cells (APC). In **chapter VI,** we show that mucosal PMN from CD patients mimic APC, that these PMN are capable of activating T-cells, and propose that in CD, enhanced activation of the T-cell response through PMN may contribute to the ongoing inflammation.

In **chapter VII,** we focus our attention on the GTPase p21Rac. Comparisons of kinome profiles of inflamed and non inflamed biopsies from IBD patients as well as non-IBD controls, show that p21Rac1 signalling is suppressed at non-inflamed sites in CD. This suppression is specific to the colonic mucosa and derives from deregulated GAP and GEF activities. 6-thioguanine (6-TG), the active metabolite of azathioprine and one of the few medications with good efficacy in CD and UC, inhibits p21Rac1 and increases innate immunity in IBD patients and healthy controls.

Finally, in **Chapter VIII** we showed that expression of the Inositol 5'-phosphatase SHIP-1 is enhanced in immune cells from CD patients, however, the activity of this phosphatase is suppressed. As SHIP-1 may modulate T-cell apoptosis, and SHIP-1 knock out mice develop IBD, these data suggest a potential role for intrinsic T-cell signalling defects in the development of CD.

Taken together, the work presented in this thesis strongly points towards a role for PMN in the development of IBD, suggests that stratifying patients according to genetic background may allow better identification of PMN defects in CD patients, and shows that in addition to their impaired innate immune function, PMN may enhance adaptive immune responses through acquisition of an APC-like phenotype.

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Chapter 2

Human granulocyte and monocyte isolation procedures: impact on functional studies

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Abstract

One of the first lines of defense against infection is the activation of the innate immune system. It is becoming clear that autoimmune disease in general and Crohn's disease in particular may be caused by a on disturbed innate immunity, and relating granulocyte and monocyte function to patient genotype has become an important part of contemporary research. Although essential to move this field forward, a systematic study comparing the efficacy and suitability for functional studies of the various available protocols for the isolation of these immune cells has not been performed. Here, we compare human granulocyte functionality upon three enrichment protocols; 1) Ficoll density gradient centrifugation, 2) anti-CD15 antibody conjugated microbeads (positive selection) and 3) Polymorphoprep™. Primary monocytes were isolated in parallel using 1) anti-CD14 magnetic microbeads, 2) non-monocyte depletion by antibody conjugated magnetic microbeads (negative selection), 3) RosetteSep™ antibody cocktail, and 4) the classical adherence protocol. Best results in terms of purity and cell functionality were obtained with positive selection by magnetic microbeads for both human granulocytes and monocytes. Whereas phagocytosis of *E. coli* bacteria was identical in all isolation procedures tested, granulocyte respiratory burst was higher in positively selected cells. In addition, different granulocyte enrichment procedures affect cell surface receptor expression to a different extent. *In toto*, we propose that positive selection of granulocytes and monocytes be adopted as the procedure of choice for studies on human granulocyte and monocyte function, but caution investigators to be aware of possible alterations in cell phenotypes upon different isolation procedures.

INTRODUCTION

Monocytes represent 3-7 percent of total white blood cells (absolute monocyte count 1.5-7 $\times 10^8$/ liter blood) in healthy human adults. Circulating monocytes, which are derived from myelomonocytic stem cells in bone marrow, have two main functions in the immune system: (1) to replenish resident macrophages and dendritic cells in peripheral tissues under normal states, and (2) to patrol healthy tissues through long-range crawling on the resting endothelium[10]. In response to inflammatory signals, monocytes quickly move to sites of infection in the tissues, engage in phagocytosis of foreign substances, and initiate an early immune response through the recruitment of neutrophils and other polymorphonuclear leukocytes (PMNs). PMNs constitute the most abundant of peripheral white blood cells, i.e. 40 to 60% (absolute count 25-750$\times 10^8$/ liter blood). Their bactericidal activity is essential in the proper clearance of infectious agents, and stems from their exocytosis of lysozyme and protease-containing granules, phagocytosis of bacteria and the concomitant production of reactive oxygen species (ROS, respiratory burst). Extravasation of PMNs to the site of inflammation in turn precedes a second wave of migrating monocytes to remove rapidly apoptotic PMNs. Monocytes, macrophages and dendritic cells are also capable of eliciting adaptive immune response via antigen presentation, a role which has also been attributed to granulocytes in inflammatory settings [6, 18]. Therefore, PMN and monocytes play a pivotal role in keeping the dynamic balance of human immune system[23].

Interest in measuring innate immune cell functionality has substantially increased, especially because of a growing acceptance of the notion that defects in innate immunity contribute to the pathogenesis of autoimmune disease in general and in particular to the pathogenesis of Crohn's disease (CD), an often severe autoimmunity towards the resident gut flora[2-5,16,20,24]. Recent genome-wide association studies (GWASs) have been instrumental in identifying novel genetic risk factors predisposing to CD and many of the alleles involved confer reduced activity in the innate immune system[5,13,17,23]. In line with these genetic studies, CD patients exhibit a phagocyte immunodeficiency that combines a primary macrophage defect and a secondary granulocytic defect [5,24]. In CD patients, dendritic cells with *NOD2* and *ATG16L1* gene mutations exhibit reduced phagocytosis and antigen presentation upon bacterial challenging [5]. Macrophage cytokine secretion in response to *E.coli* loading is impaired in CD[20]. Furthermore, after acute trauma in the gut mucosa and skin, PMN recruitment, bacterial clearance and ROS production are attenuated[16,19,21]. Taken together, the body of contemporary biomedical literature strongly supports the concept that innate immune cell dysfunction is associated with the pathogenesis of CD-like and other autoimmunity [24], triggering investigations into the properties of innate immune cells in patients, and comparison of their phenotype to the genotype of risk genes relevant for autoimmunity. The results of such studies may well depend on the protocols employed for isolation of the immune cells. A comparison of both the yield and specificity of the available protocols for monocyte and PMN isolation from patient peripheral blood, as well as the relative performance of cells isolated using these protocols in subsequent functional experimentation, is urgently called for.

These considerations prompted us to perform a systemic evaluation of the most frequently used methodologies for the isolation of granulocytes and monocytes from human peripheral blood. PMN were isolated in parallel using (1) Ficoll density gradient centrifugation, (2) polymorphprep™ density gradient centrifugation, and (3) anti-CD15 antibody conjugated magnetic microbeads (positive selection), after which PMN functionality was assessed by *in vitro* phagocytosis and ROS production assays. Monocytes were isolated from peripheral blood in parallel using (1) anti-CD14 antibody conjugated magnetic microbeads (positive selection), (2) non-monocyte depletion by antibody conjugated magnetic microbeads (negative selection), (3) immunorosette based RosetteSep™ antibody cocktail (RosetteSep™), and (4) adherence, aiming to assess their suitability for *in vitro* phagocytosis analysis. We conclude that positive selection of granulocytes and monocytes by anti-CD15 and anti-CD14 antibody conjugated magnetic microbeads, respectively, are best suited for studies in which purity is imperative, but that in general the isolation method of choice should depend on the type of functional assay to be used.

MATERIALS AND METHODS

Granulocyte isolation from human peripheral blood

Heparin and EDTA anti-coagulated blood was obtained from healthy volunteers. Neutrophils were isolated as described previously[9]. In short, mononuclear cells were removed by centrifugation of heparinized blood over Ficoll-Paque (Amersham), followed by erythrocyte lysis with ice-cold NH4Cl solution. For positive selection, granulocytes obtained from Ficoll density gradient centrifugation were subsequently subjected to anti-CD15 microbead isolation (Miltenyi Biotech, Amsterdam, the Netherlands), using manual columns, according to the manufacturers' instruction. Additionally, PMN were isolated from EDTA anti-coagulated blood using PolymorphPrep™ (Axis-Shield, Norway). When present, erythrocytes were lysed with ice-cold NH_4Cl solution. Before functional testing, PMN were allowed to recover for 30 minutes at 37°C in RPMI 1640 supplemented with 0.5% human serum albumin (HSA; Sanquin, The Netherlands). Cells were resuspended in incubation buffer (20mM HEPES, 132mM Nacl, 6mM KCl, 1mM $MgSO_4$, 1.2mM KH_2PO_4, 5mM glucose, 1 mM $CaCl_2$, and 0.5% HSA) prior to subjecting them to functional assays. All isolation procedures were done in parallel per healthy donor.

ROS production assay

ROS production was performed as described [8]. Shortly, PMN ($2x10^6$ cells/ml) were incubated with DHR 123 for 15 minutes and stimulated with 1μM fMLP for 30 minutes. For priming experiments, cells were pretreated with 5ng/ml GM-CSF for 15 minutes prior to N-formyl-methionine-leucine-phenylalanine (fMLP) stimulation. Stimulation of PMN with fMLP was terminated by washing the cells with ice-cold PBS containing 1% HSA and placing them on ice. Oxidation of Dihydrorhodamine 123 (DHR123) to the fluorescent Rhodamine 123 was measured by FACS-analysis within 30 minutes after termination of stimulation.

Monocyte isolation from human peripheral blood

Heparinized blood was obtained from healthy volunteers after informed consent. In order to optimize monocyte isolation method, monocytes from the same healthy donor were isolated by four different strategies in parallel. In short, monocyte positive selection by monoclonal CD14 antibody (isotype: mouse IgG2a) conjugated microbeads (Miltenyi Biotec, Germany) and monocyte negative selection by monocyte isolation kit II (Miltenyi Biotec) were performed with manual columns strictly according to the manufacturer's protocol, as was monocyte isolation by RosetteSep™ (Stemcell technologies, France). Monocyte isolation by adherence method was performed as described previously[7]. Briefly, peripheral blood mononuclear cells were isolated immediately after collection using Lymphoprep™ gradients (Axis-Shield PoC As, Norway). Monocytes were further enriched by virtue of their attachment to a culture plate for 2 hours, washed 3 times with warm phosphate buffered saline (PBS) to remove non-adherent cells, the adherent monocytes were recovered by cell scraper. The purity of monocytes was evaluated by fluorescent staining with CD14-PE monoclonal antibody (UCHM1, murine IgG2a, IQ products, Netherlands) and FACS analysis. The recovery of monocytes was evaluated by Trypan blue staining and counted using a Zeiss microscope.

Cell culture

Monocytes were cultured in complete medium consisting of RPMI1640 (PAA laboratories, Austria) supplemented with 10% heat-inactivated FCS (PPA) and 10 µg/ml Gentamicine (Centrafarm, Netherlands) at 37°C in 5% CO_2 humidified air.

Phagocytosis assay

GFP expression vector was transformed into *E.Coli* and grown in LB media until OD of 400 was reached. Bacteria were fixed using 4% formaldehyde. Isolated PMN were resuspended (2 x10^6/ml) in RPMI containing 10% FCS and 1x10^6 bacteria were subjected to PMN phagocytosis at 37°C for 15 minutes. The percentage of phagocytosing PMN was determined by flow cytometry by analyzing the percentage of GFP positive PMN. Phagocytosis at 0°C was used as negative control for each experiment. No FITC positive PMN were observed under this condition, confirming active phagocytosis of bacteria rather than attachment of bacteria to PMN membranes. Alternatively, 4% formaldehyde-fixed *E. coli* were labeled with FITC fluorescence by incubation in 1mg/ml FITC solution containing 0.1M Na_2CO_3 pH 9.5 for 1 hour followed by complete washing. The efficiency of labeling was tested by FACS analysis (see below). Bacterium concentration was quantified using Quantimet HR550 image analysis software (Leica) to analyze microscopic images of FITC-*E. coli* taken with a Leica (Wetzlar, Germany) DMRXA epifluorescence microscope[25]. For monocytes, the phagocytosis assay was performed according to Mandell and Hook[15]. In short, monocytes (1× 10^4/ml) were incubated with FITC-*E. coli* at a 1:5 ratio for 5 or 15 minutes at 37°C in RPMI1640 medium containing 10% heat-inactivated FCS. Thereafter, phagocytosis was evaluated microscopically by counting both the number of monocytes exhibiting phagocytosis and the number of bacteria phagocytosed per

monocyte. At least 300 cells were counted for each slide. The engulfment rather then attachment of *E. coli* was confirmed by confocal microscope and cultures at 4 ^{o}C.

FACS analysis

Isolated PMN or whole blood after red cell lysis were stained resuspended in PBS/EDTA/1%FCS). After blocking with FcR blocking reagent from Miltenyi Biotech (Amsterdam, the Netherlands) for 15 minutes, cells were labeled with anti-CD14-PerCP-Cy5.5 (clone M5E2), anti-CD66b-PerCP-Cy5.5 (G10F5), anti-CD15-PE (clone HI98), anti-TLR2-Alexa 647 (clone TL2.1), anti-TLR4-PE (clone HTA125) (all from Biolegend, San Diego, CA). No FcR blocking was performed when staining for FcReceptors using anti-CD16-PE (clone 3G8, Biolegend), anti-CD64-PE (clone 10.1, Biolegend) or anti-CD32-FITC (clone FL18.26 from BD Bioscience, Franklin Lakes, NJ). After washing with PBS, fluorescence was measured by flow cytometry.

Analysis of purity

Analysis of the purities of granulocytes and monocytes by FACS was performed using PerCP-Cy5-labeled CD66b (Clone G10F5, Biolegend) and PE-labeled CD14 monoclonal antibody (clone UCHM1, murine IgG2a, IQ products, Netherlands), respectively. In short, cells were incubated with appropriate antibodies (10 µl per 5 × 10^5 cells) for 30 minutes on ice. Cells were washed twice in PBS/0.1% BSA before FACS analysis. For identifying the platelet contaminants and observing the cytoskeleton of monocytes, enriched monocytes were further stained intracellularly with TRITC conjugated phalloidin (Sigma-aldrich). In short, cells were fixed in PBS/4% formaldehyde for 10 minutes, washed twice in PBS, permeabilized in PBS/0.1% Triton X-100 (Sigma-Aldrich), treated with TRITC conjugated phalloidin (25µg/ml) for 30 minutes in dark, and washed three times in PBS. Platelets were identified by their morphology under fluorescence microscopy. Contaminating cells in granulocyte isolates were discriminates using anti-CD19-PE, clone IB19 (B-cell), anti-CD3-Amcyan clone SK7 (T-cell) and anti-CD14-PerCP clone M5E2 (Monocyte) antibodies. DAPI was used to exclude dead cells.

Statistics

ROS production (fMLP vs GM-CSF+fMLP), priming thereof, phagocytosis, MFI of cell surface expression were compared by student T-test for paired samples. All experiments were performed for at least 3 individual donors.

Results

Comparison of three commonly used isolation protocols for purity of enriched granulocytes.

PMN were isolated from blood from healthy volunteers using, in parallel, three of the most commonly used methods in literature. Purity of isolates was analyzed by flow cytometry, and proved to be 96.5, 99.7 and 90.8% of nucleated cells for Ficoll, anti-CD15 microbead positive selection and Polymorphprep^{TM} isolation methods, respectively (Figure 1A, Table 1). This measure, however, excludes non-nucleated cells (erythrocytes and thrombocytes) as well as ghosts derived from necrotic cells.

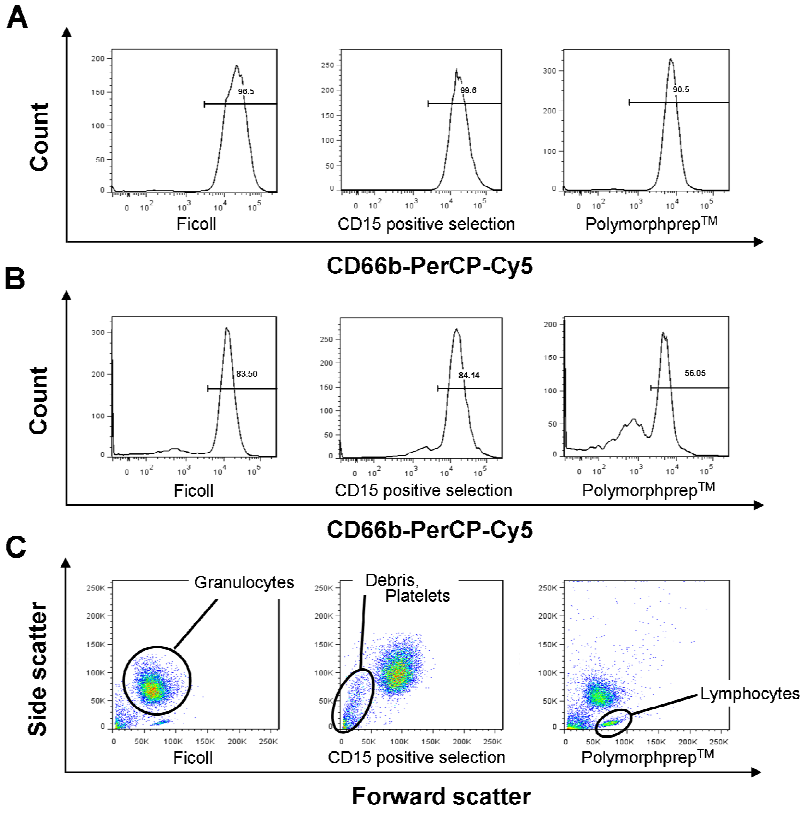

Figure 1: ***The purities of enriched granulocytes.*** (A) Representative flow cytometry histograms (of n=4) showing the purity of enriched granulocyte isolates, obtained by either 1) Ficoll density centrifugation, 2) anti-CD15 antibody conjugated magnetic microbeads (positive selection), 3) Density centrifugation using Polymorphprep™. Histograms represent CD66b positive cells after gating for nucleated cells using forward and sidescatter profiles . (B) The purities of granulocytes after isolation are expressed as fraction of all cell-like particles (including nucleated cells, non-nucleated cells, and ghosts) after enrichment. (C) Forward and side scatter plots of the enriched samples showing the nature of contaminants as identified using traditional forward and side scatter morphology.

Hence, we also determined the percentage of CD66b positive cells as a function of all cell-like particles (i.e. all particles in which Ø >0,5 µM). When results were expressed this way, Ficoll density gradient centrifugation yielded 85% granulocyte purity, compared to 85% for positive selection (see Figure 1B for representative example). Polymorphprep™ isolation method resulted on average in 68% purity, largely due to a higher amount of contaminating debris particles and lymphocytes (Figure 1C). Also, in our hands, the highest variability in purity between isolations was observed when using Polymorphprep™, suggesting that this method is somewhat more sensitive to small day to day variations. We also noticed a donor to donor variation in adherence of

the lymphocyte ring to the plastic disposables after centrifugation, which may account for some of the contamination variability observed. When analyzing the nature of this contaminating mononuclear cell fraction, these were found to be mainly CD3-positive T-cells, with little or no $CD19^+$ B-cell or $CD14^+$ monocytes (not shown). PMN are amongst the most short-lived cells in the body. They are extremely fragile cells, and prone to apoptosis upon withdrawal from the blood. Importantly, however, no more than 3.7% dying cells were identified by 7AAD (Fig 3, first graph) or DAPI staining (not shown) as determined by FACS analysis of any of the isolation methods used, indicating that no significant cell death was induced during isolation procedures. In conclusion, the best results in terms of granulocyte purity were obtained using anti-CD15 microbead positive selection methods.

Methods	Yield ($10^{5)}$	Purity (%)	Procedure time	Cost (€/10ml blood)
Ficoll centrifugation	13.5±6.4	96.5±1.8	1.5 hours	5.4
Positive selection	5.1±4.3	99.7±0.1	2.5 hours	33*
Polymorphprep™	12.7±3.6	90.8±5	1 hour	6.1

Table 1: ***Comparison of the yield and purity of granulocytes, time consumption and costs of the three different isolation procedures evaluated in the present study***.
Note: The cost for positive selection did not include the magnetic separator. Purity shown is of total nucleated cells. *Cost of positive selection depends on the yield of PMN after Ficoll centrifugation, and shown is the amount calculated based on 10^7 total cells. Mean ± SD is shown of 4 independent experiments. Yield of PMN isolated by positive selection was significantly lower compared to Ficoll centrifugation and Polymorphprep (P=0.015 and 0.003, respectively, by student T-test). Purity of PMN isolated by Polymorphprep was significantly lower than per Ficoll centrifugation or Positive selection (P= 0.033 and 0.041, respectively, by Student T-test).

Comparison of granulocyte yield obtained using the three most commonly used PMN isolation protocols

The absolute recovery rates of PMN after the three isolation procedures are shown in Table 1. Typically, isolation of PMN by positive selection, though leading to the highest purity, also yielded the lowest recovery rates; 5×10^5/ml peripheral blood were obtained, which was significantly less then the yields obtained using either Ficoll centrifugation (13.5×10^5/ml peripheral blood) or Polymorphprep™ (12.7×10^5/ml peripheral blood). Variation in yield was slightly higher using Ficoll isolation method, but as this did not affect purity and this method also results in the highest yield of all methods tested, we did not consider this to be a negative trait. In fact, having a purity of 96.5% and the highest overall yield, we would recommend granulocyte enrichment by Ficoll centrifugation when high numbers of cells are required.

Granulocyte functionality in a ROS production assay following different isolation protocols

PMN are easily activated by trace amounts of bacterial lipopolysaccharides or mechanical stressors. To determine whether our different isolation methods give unwanted pre-activation (priming) of PMN, we studied their ROS production.

Spontaneous PMN ROS production was not different between isolation procedures (data not shown). ROS production can be triggered by the bacterial peptide analogue fMLP, and fMLP-induced ROS production is significantly enhanced in GM-CSF-primed PMN. As shown in Figure 2A, ROS production in PMN isolated by Polymorphprep™ showed a lag in fMLP-induced ROS production when compared to either Ficoll or positive selection methods (115±10% vs 154±27 and 168±43, respectively, at t=5 min, up to 201±58% vs 235±55 and 253±65, respectively, at t=30 min). When we compared fMLP-stimulated ROS production in GM-CSF-primed PMN, no significant increase in ROS production was observed in PMN isolated by Polymorphprep™ (Figure 2A, left graph) due to high variability between experiments (n=4). In contrast, Ficoll-isolated PMN showed significant priming of FMLP-induced ROS production, and an even higher priming of ROS production was observed in PMN isolated by positive selection (Figure 2A and 2B). These results suggest that the ability of PMN to be primed for ROS production is highest in PMN isolated by positive selection, which may argue for lower activation status upon isolation in these cells. (Figure 2B). Therefore, for ROS production analysis, positive selection of PMN may be the best option.

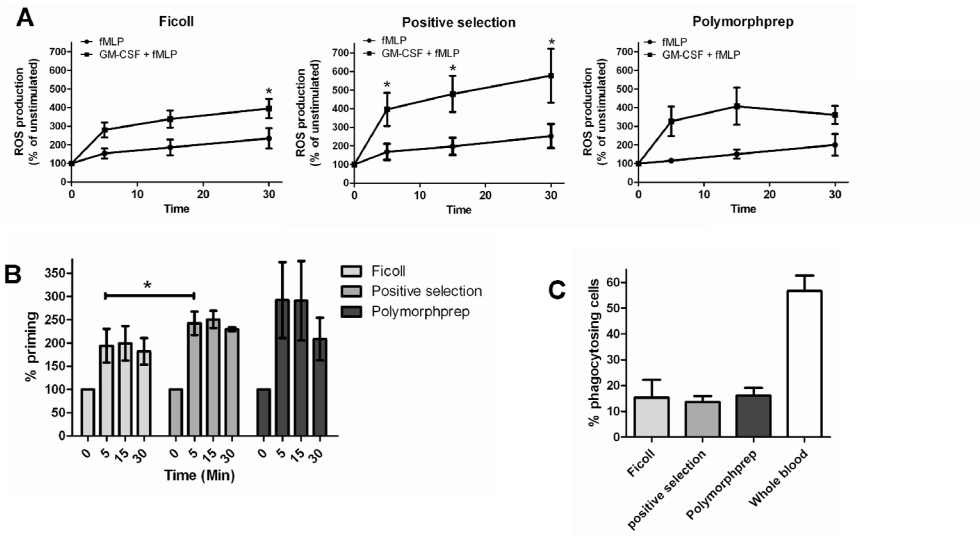

Figure 2: ***Functional testing of enriched granulocyte fractions.*** (A) Granulocytes isolated by Ficoll density centrifugation, anti-CD15 antibody conjugated magnetic microbeads (positive selection) or density centrifugation using Polymorphprep™ were subjected to ROS production analysis. Stimulation with fMLP results in low production of ROS, indicative of resting cells, whereas priming of cells with GM-CSF yields the highest fMLP-induced ROS production in granulocytes isolated by CD15- microbeads positive selection. Mean ± SEM of 4 experiments is shown. Asterisks indicate significantly higher ROS production in GM-CSF+fMLP stimulated cells as compared to fMLP alone ($P<0.05$). (B) Priming capacity was determined by expressing ROS production in GM-CSF cells as a percentage of ROS production in unprimed, fMLP-stimulated granulocytes (Mean ± SEM of 4 experiments). (C) Enriched granulocytes were challenged with FITC-expressing *E.Coli* for 15 min, after which FITC fluorescence of isolates was determined by FACS analysis. Whole blood was used as positive control (Mean ± SEM of 4 experiments).

Granulocyte functionality in an Escherichia coli phagocytosis assay following different isolation protocols.

Ficoll has been shown to change PMN shape and migratory capacity, indicating an effect on cytoskeletal rearrangement[11]. Phagocytosis is dependent on the actin cytoskeleton, and as such may be affected by Ficoll isolation procedures. Positive selection of neutrophils relies on the antibody binding of CD15, a carbohydrate adhesion molecule. Ligation of CD15 antibodies to this integrin-associated molecule may potentially affect phagocytosis and adhesion of PMN[14]. We therefore studied the *E. coli* phagocytosing capacity of PMN isolated by different methods, using phagocytosis of PMN in whole blood as control. To distinguish PMN in whole blood samples, cells were stained for CD15, and the percentage of PMN containing GFP-positive *E.Coli* was determined by FACS analysis. As expected, phagocytosis of non-isolated PMN is more efficient than that of isolated granulocytes, with more than 56% of granulocytes taking up GFP-positive bacteria. No significant differences in the percentage of phagocytosing cells were observed in PMN isolated by either Ficoll centrifugation, positive selection or Polymorphprep™ (15.3%, 13.6% and 16.1%, respectively, n=4; Figure 2C). Engulfment of bacteria rather then adhesion to PMN cell surface was confirmed by confocal microscopy (supplementary movie 1). In addition, the amount of bacteria taken up per cell, as determined by mean fluorescence intensity (MFI) of GFP-positive PMN, did not differ per isolation method used (MFI of 1898, 1656 and 1973 for Ficoll centrifugation, positive selection or Polymorphprep™, respectively, not shown).

Expression of PMN cell surface markers following different isolation procedures.

Isolation of granulocytes may affect their expression of cell surface receptors and other molecules, and thereby alter specific granulocyte functions. CD15 and CD66b are adhesion molecules involved in PMN phagocytosis and chemotaxis. Analysis of CD15 expression upon different isolation procedures reveals a slight but significant decrease in CD15 and CD66b expression upon Polymorphprep™ isolation as compared to Ficoll isolation and positive selection, respectively. Although this is not likely to affect phagocytosis (as seen above), it is conceivable that other, untested functions (e.g. migration), could potentially be affected. We also assessed expression of the CD64 FcγRI, the CD32 FcγRII and the low affinity FcRIII, CD16. CD64 expression was significantly higher in positively selected PMN as compared to PMN isolated by Ficoll. In addition, cells isolated by positive selection showed a small but significant increase in CD16 expression as compared to Polymorphprep™ isolates. No differences in CD32 expression were observed between isolation protocols. Next, we analyzed the expression of Toll like receptor (TLR) 2, TLR4 and co-receptor CD14, which bind lipopolisaccharise (LPS), the major component of the outer membrane of Gram-negative bacteria such as *E. coli*[12]. A dramatic increase in TLR2 and TLR4 expression was observed on PMN isolated by positive selection. This could have substantial consequences for studies into the function of these receptors as well as studies into functional LPS responses, and isolation procedures should therefore be carefully considered by investigators undertaking such studies.

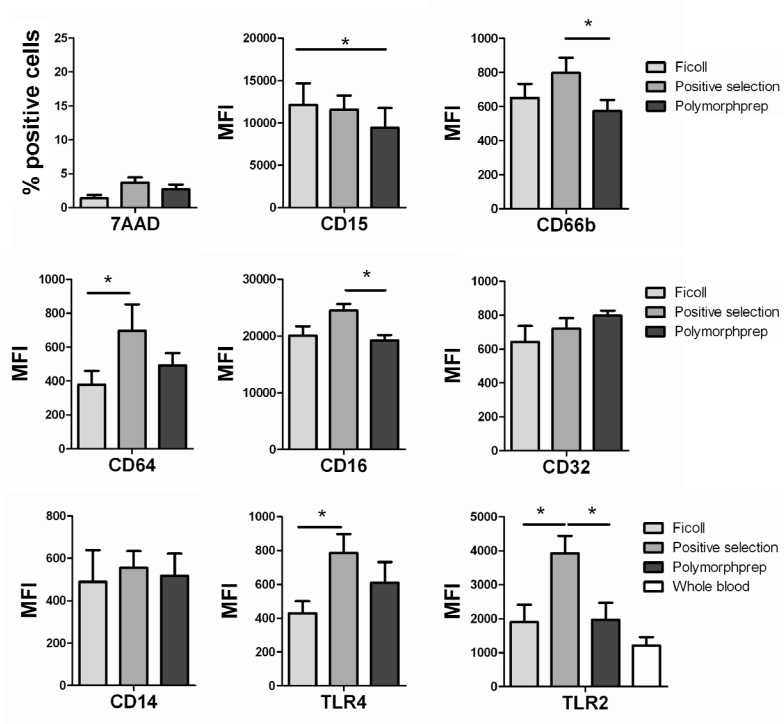

Figure 3: ***Expression of cell surface markers on isolated granulocytes.***
Granulocytes isolated by Ficoll density centrifugation, anti-CD15 antibody conjugated magnetic microbeads (positive selection) or density centrifugation using Polymorphprep™ were subsequently stained for 7AAD, CD15, CD66b, CD64, CD16, CD32, CD14, TLR2 and TLR4. Except for 7AAD staining, which was present in less then 5% of PMN, all other markers were present on >98% of all isolated cells, and the median fluorescence intensities of these markers was determined. Mean ± SEM of 6 experiments is shown.

Methods	Yield(10^5)	Purity (%)	Procedure time hours	Cost (€/10 ml blood)
Positive selection	2.2±0.3	98.5±0.5	2.5	23
Negative selection	2.2±0.4	97±0.8	3	26
Adherence	2.0±0.7	68.1±3.4	4	8
RosetteSep®	4.4±0.8	63.7±4.9	1.5	46

Table 2. ***Comparison of the yield and purity of monocytes, time consumption and costs of the four different isolation procedures evaluated in the present study.***

Note: The cost for positive selection and negative selection did not include the magnetic separator. The yield of monocytes enriched by adherence was shown for the optimal washing condition. Purity shown is of total nucleated cells. Mean of 4 experiments ± SD is shown. The yield and purity of monocytes were significantly different among procedures (P=0.0039 and P=0.018, respectively by ANOVA analysis).

Comparison of the four most commonly-used isolation protocols for purity of enriched monocyte cultures

Monocytes of healthy individuals were isolated from peripheral blood in parallel by the four procedures most commonly used in the literature. First we decided to assess the purity of the monocyte fraction following the four different types of enrichment as a percentage of all nucleated cells using FACS. Using this measure, it was observed that monocytes were obtained with purities of 98.5%, 97.0%, 68.1% and 63.7% by positive selection, negative selection, adherence and RosetteSep™, respectively (Fig. 4A). Furthermore, visual inspection of monocyte cultures revealed the presence of non-nucleated particles. When results were expressed as percentage of all recorded events (Ø >0,5 µM) the monocyte purity obtained was 95.4%, 48.7%, 58.5% and 35.9% for respectively positive selection, negative selection, adherence and RosetteSep™ (Fig. 4B, representative example). Thus with regard to purity, positive selection yields the bests results for monocyte isolation, both judged by the monocyte fraction of all nucleated cells and as fraction of all cell-like bodies. Importantly, after positive selection procedures during which monocytes were labeled with antibodies for CD14 receptors, abundant CD14 expression was still detected by anti-CD14 PE, indicating a functional condition of these cells with respect to CD14 (Fig. 4D). Next, we analyzed the nature of the contaminants in the enriched samples obtained using the four different isolation procedures. As shown in Fig. 4C, compared to samples obtained through positive selection, large amounts of platelets remained in the enriched monocyte cultures after negative selection. After adherent isolation procedure, the major contaminants are lymphocytes and platelets. After RosetteSep™ procedures, we observed large amounts of lymphocytes, platelets, and non-specific cellular aggregates, which significantly compromise the purity of this monocyte culture.

Comparison of monocyte yield obtained using the four most commonly-used monocyte isolation protocols

The absolute recovery rates of monocytes after the four isolation procedures are depicted in Table 2. The purities and recoveries of monocytes after positive selection, negative selection, and RosetteSep™ procedures were highly reproducible. During the enrichment procedure by adherence, due to the difficulties to standardize the washing steps by which monocytes and lymphocytes are separated, outcomes were more variable, at least in our hands. In this respect, we observed that this methodology is sensitive to inadequate washing of cultures resulting in significantly compromised monocyte purity, whereas excess washing has substantial consequences with respect to monocyte recovery yield (not shown). Nevertheless, typically, isolation by adherence enriched monocytes to a purity of 68.1% and a recovery of 2.0×10^5/ml peripheral blood, which is not markedly different from the yields obtained using either positive or negative selection. The RosetteSep™ procedure leads to substantially better results in this respect and takes less time, but the cultures obtained suffer from impurities (see above) and on a per monocyte basis, it remains slightly more expensive (Table 1). Viability of monocytes in all isolates was >99%, as determined by fluorescence microscopy of DAPI stained nuclei (for example, see Fig. 4D).

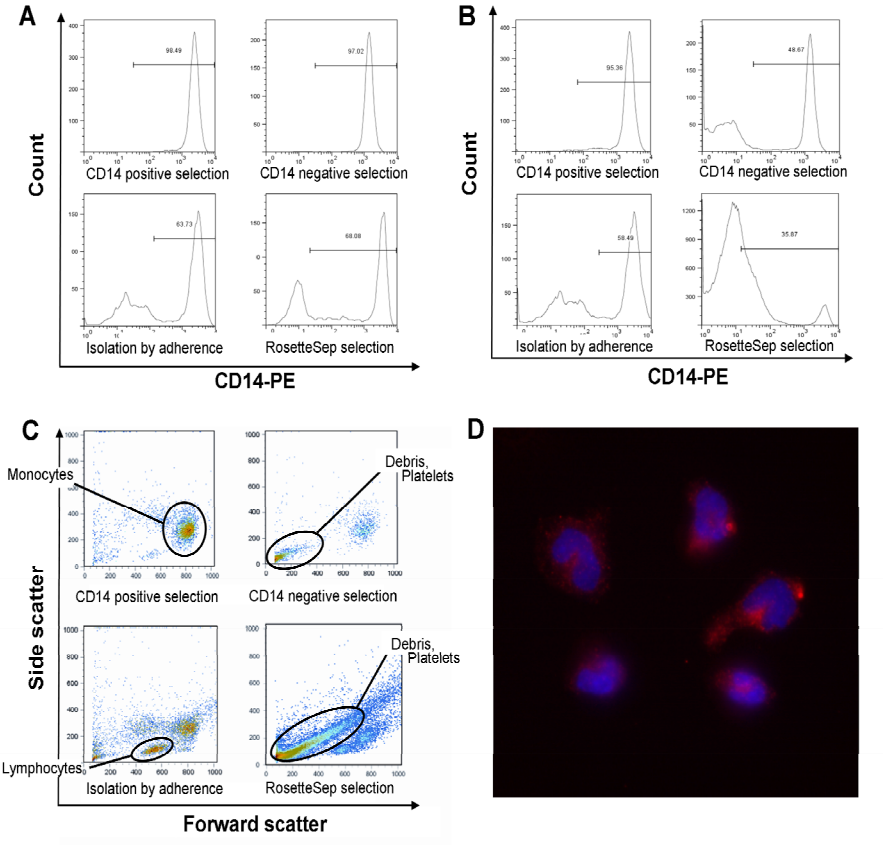

Figure 4: ***The purities of enriched monocytes.*** (A) Representative flow cytometry histrograms (of n=3) showing the purity of enriched monocyte cultures, obtained by either 1) anti-CD14 antibody conjugated magnetic microbeads (positive selection), (2) non-monocyte depletion by antibody conjugated magnetic microbeads (negative selection), (3) a classical adherence protocol, and (4) immunorosette based RosetteSep™ antibody cocktail, as percentage of monocytes out of nucleated cells. (B) The purities of monocytes after isolation are expressed as fraction of all cell-like particles (including nucleated cells, non-nucleated cells, and ghosts) after enrichment. (C) Analysis of the nature of contaminants in the enriched samples as identified using traditional forward and side scatter methodology. (D) Monocytes purified by positive selection were stained for CD14 and nuclei using anti-CD14 PE antibody (red) and DAPI (blue).

Monocyte functionality in a Escherichia coli phagocytosis assay following different monocyte isolation protocols

Along with TLR4, CD14 acts as a co-receptor on monocytes, detecting bacterial LPS. In order to evaluate the effect of CD14 antibody binding during positive selection procedures on phagocytic capacity of enriched monocytes, we challenged monocytes enriched by positive selection or negative selection with FITC-labeled *E. coli.* Phagocytosis index is significantly influenced by bacteria/monocyte ratio (Zhou L, et al, unpublished data). For the studies aiming to compare (Fig. 5B). The engulfment of *E.coli* by monocytes rather than attachment to the cell surface was confirmed by

confocal microscopy (Fig. 5C and Supplementary movie 2). Importantly, we also observed active phagocytosis of platelets and ghosts (Supplementary Fig. 1), potentially suggesting competing effects between *E. coli* and platelets and stressing the importance of an isolation procedure that does not yield such contaminants in the culture. Therefore, *in toto*, the anti-CD14 antibody-conjugated magnetic microbeads (positive selection) protocol appears the best suited monocyte isolation method for this type of phagocytosis analysis.

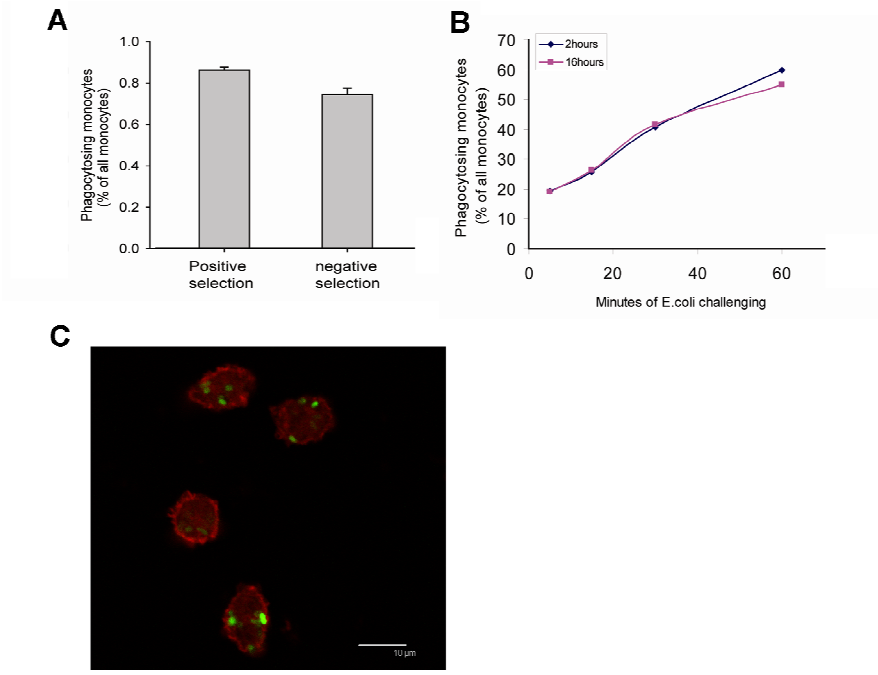

Figure 5: ***Phagocytic capacity of monocytes***. (A) Monocytes obtained through the positive selection and negative selection procedures were challenged with FITC-labeled *E. coli* for 5 minutes, in parallel. Phagocytosis of *E. coli* is expressed as the percentage of monocytes displaying phagocytosis. *E. coli* phagocytosis of monocytes after the two isolation procedures did not show significant difference, although the level of phagocytosis in negative selection group is slightly lower (n=3, $p>0.05$, values were presented with Mean±SEM) (B) After positive selection procedure, monocytes after 16 hour culture exhibit the same *E. coli* phagocytotic capacity compared to the 2 hour culture (representative of 3 independent experiments). (C) A representative trans-sectional image of monocytes by confocal microscope confirming that *E. coli* are engulfed rather by attached to monocytes, red: cytoskeleton of monocytes (F-actin) stained by TRITC-phalloidin; green: FITC-labed *E.coli*.

DISCUSSION

For many studies, including those on the involvement of granulocytes or monocytes in the pathogenesis of autoimmune diseases, isolation of a pure population of cells is essential. Although all four monocyte isolation procedures tested in this study yielded functional phagocytosis-competent monocytes, results with respect to purity and recovery are markedly different for the different procedures. We view especially purity as a concern, as this may be important for functional studies. We observed when challenging monocyte cultures with FITC-labeled *E. coli*, that contaminating lymphocytes markedly compromise the accuracy of phagocytosis quantification. This was due to two reasons: first, as a result of the extremely similar morphology of monocytes and lymphocytes, quantifying the percentage of phagocytosing monocytes by fluorescence microscopy is markedly disturbed by lymphocyte contamination. Second, we observed that lymphocytes are also able to adhere *E. coli* to their cell surface (data not shown), which may further hamper quantification. This is especially obvious when monocytes are mixed with a large population of lymphocytes (eg. PBMC) or a large amount of bacteria is loaded. Furthermore, we observe that co-purified thrombocytes and cell ghosts are also subject to phagocytosis, with unknown effects on monocyte physiology and thus possibly compromising experimentation. In addition, it has been shown that isolating a pure cell population is important for other functional studies of monocytes, e.g. antigen presentation and cytokine production. For these reasons, isolation of monocytes employing positive selection and anti-CD14 conjugated microbeads appears the technology of choice, at least for phagocytosis analysis. However, due to the high costs of commercial reagents and required instruments for positive selection, in studies where the purity of monocytes is not strictly required, traditional adherent isolation procedures remain an option. For instance, in studies using monocyte-derived dendritic cells, the lymphocyte contaminants may be less important as the latter will not survive for longer than 1 week in the absence of IL2.

With respect to the absolute yields obtained, the RosetteSep™ comes out superior, but does not yield pure cultures. The other technologies are comparable, including the traditional adherence protocol, the latter is subject to substantial experimentator- and day-to-day-dependent variability, hampering its application and comparison of results. Negative selection yields less pure cultures and is more expensive per monocyte and thus appears to be a less attractive choice.

Regarding PMN isolation, comparison of three widely used methods showed that isolation by positive selection yielded isolates with the highest purity, followed closely by Ficoll centrifugation. Polymorphprep™ isolates showed the highest impurities, which can be problematic in certain experiments. Another considerable drawback in the use of Polymorphprep™ is that relatively high purities can only be reached when using EDTA as anticoagulation agent, which may not always be practical. Theoretically, the main advantage of Polymorphprep™ is the elimination of erythrocyte lysis steps. However, red blood cell contamination of the PMN ring is often observed (up to 6% of isolate, as per manufacturer's datasheet), and a mild yet potentially cell-activating lysis step may therefore nonetheless be required.

Isolates from positive selection methods showed the highest ROS production in response to GM-CSF + fMLP treatment, which may imply that this method of isolation induces the least desensitization of PMN. Priming capacity of GM-CSF was reduced in PMN isolated by Polymorphprep™, presumably due to the variability in quality of the isolates. Hence, positive selection seems the method of choice for studying ROS production, closely followed by Ficoll isolation. ROS production is dependent on the actin cytoskeleton, in that disruption of actin polymerization results in increased ROS levels [25]. As CD15 ligation may affect cytoskeletal rearrangement, it is theoretically possible that positive selection using CD15 antibodies can enhance ROS production or affect phagocytosis. However, phagocytosis of *E. Coli* was similar in all three methods tested, and fMLP-induced ROS production in positively selected isolates was equal to that in isolates from Ficoll centrifugation, indicating that CD15 engagement in positive selection procedures at least does not affect these functions. Our results did clearly show an upregulation of the toll like receptors TLR2 and TLR4 on PMN isolated by positive selection. Although this is not likely to affect either the ROS production or *E.Coli* phagocytosis tested here, this increase may nevertheless influence other cellular assays investigators into innate immunity may want to study. Careful consideration of different neutrophil isolation techniques is therefore required for every functional study considered by investigators, and may depend on the type of assay desired.

In this study, we have compared 4 widely used monocyte isolation procedures, and 3 commonly used granulocyte isolation methods. However, other isolation procedures have been described that were not covered in this study. For instance, monocytes may be isolated by CD14-positive selection in the magnet-based MagCellect system from R&D Systems. However, this company does not at this time offer granulocyte isolation kits. In addition, using Elutra™ separator, isolates by which monocytes are enriched on the basis of size and to a lesser extent density from an entire apheresis product have been described to result in approximately 75% purity of monocytes. However, this procedure requires an automated system, and may therefore not be suitable for every laboratory. For granulocytes, EasySep® magnetic isolation kits based on CD66 expression are available from Stemcell technologies. How these different isolation procedures (CD15 versus CD66b positive selection) affect PMN cell function remains to be investigated.

In conclusion, if high purity of cells is required, and limited cells are needed, we would consider that PMN and monocyte isolation using positive selection is the most suitable method. Where high purity of PMN or monocytes is not strictly required, Ficoll density gradient centrifugation and the traditional adherent isolation procedure remain good options for PMN and monocytes purification, respectively. However, investigators into innate cell functions should be well aware of possible alterations in cell phenotypes upon different isolation procedures, and the best isolation procedure may depend on the assays planned to be used.

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Supplementary material:

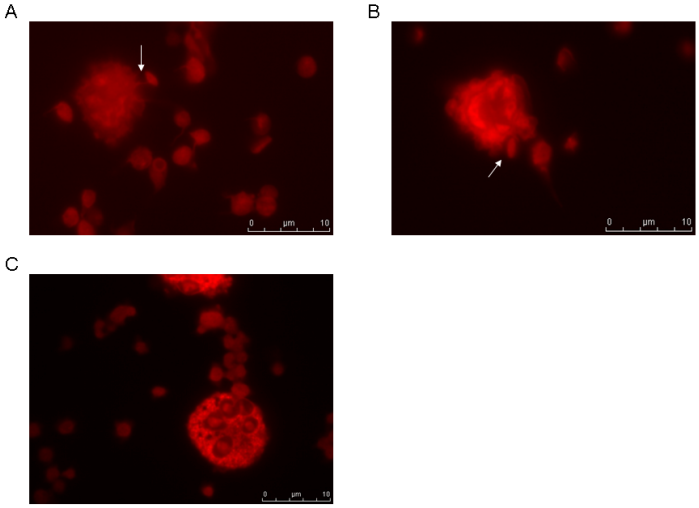

Figure S1: ***Illustrations of active phagocytosis by monocytes of contaminating platelets.*** Monocytes were isolated by traditional adherent protocol with extra 10-minute centrifugation during the Ficoll separation and its washing steps. The extra centrifugation caused more platelet contamination. The arrow in (A) shows the detection of platelets by filopodia of monocytes. The arrow in (B) shows the site of engulfment of platelets. (C) phagocytosing monocyte.

Supplementary movie 1: ***Z-stacks of confocal microscopy of phagocytosing PMN.***
PMN isolated by Ficoll density centrifugation were incubated with GFP-expressing bacteria. Nuclei were stained with DAPI, and confocal microscopy was performed. (Refer: Compact disc)

Supplementary movie 2: ***Z-stacks of confocal microscopy of phagocytosing monocytes.***
Monocytes isolated by CD14 positive selection procedure were incubated with FITC-labeled bacteria. Monocytes were stained with phalloiding-TRITC to detect actin cytoskeleton, and confocal microscopy was performed. (Refer: Compact disc)

Chapter 3

Altered neutrophil functionality and cell signalling in Crohn`s disease with skin manifestion

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Submitted

Chapter 4

Linking risk conferring mutations in NCF4 to functional consequences in Crohn`s disease

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We read with interest the paper from Muise *et al.* in which they describe a rare variant in the NCF2 gene, which demonstrates a diminished RAC2 binding capacity[1]. The NCF2 encoded protein $p67^{phox}$ is one of the components of the NADPH oxidase complex which drives the production of reactive oxygen species (ROS) during the bactericidal response of innate immune cells. Disturbed granulocytic ROS output as a result of impaired functioning of this enzyme complex has been shown in a number of diseases, including MDS and chronic granulomatous disease[2,3]. As Muise and colleagues point out, these diseases have been linked to development of a colitis resembling that observed in Crohn's disease (CD), suggesting a potential role for impaired ROS production in CD pathology.

Genome wide association studies (GWAS) are a promising tool to identify genetic variants of genes linked to an increased risk of developing CD. Amongst the single nucleotide polymorphisms (SNPs) identified so far is a T to C substitution in intron 1 of NCF4, encoding the $p40^{phox}$ NADPH protein. Association of this SNP, rs4821544, with ileal CD was confirmed by Muise *et al*. However, although in general much is made of the importance of GWAS-identified risk conferring SNPs in patients, it remains as yet unclear how these SNPs affect patient cell function, as functional studies are mostly lacking. Although Muise and colleagues very clearly show that a non-synonymous mutation in NCF2 affects its interaction with RAC2, their paper shows no direct proof that this affects granulocytic ROS production. Although making a good case, the fact that the c.113 G→A mutation is so rare (they were only able to measure ROS in one patient bearing this mutation), impedes conclusions as to its general role in CD occurrence.

To further address the potential of SNP variants in NADPH oxidase genes to confer a functional consequence in CD, we have investigated granulocyte ROS production in CD patients bearing either the NCF4 risk allele (C), or patients homozygous for the non-risk allele (T). Patient characteristics (age, treatment, disease location, gender, fistulisation) were identical between the two groups (not shown). We observed no differences in fMLP-triggered intracellular ROS production between carriers and non-carriers of the risk allele. However, fMLP-induced ROS production was significantly lower in GM-CSF primed neutrophils from CD patients with an NCF4 mutation (Figure 1). These results are consistent with previous studies, showing that $p40^{phox}$ is important for intracellular ROS production in response to certain triggers such as phagocytosis, but plays a smaller role in PMA or fMLP-induced ROS production[3]. We observed no differences in granulocyte respiratory burst when patients were stratified according to ATG16L1 (rs10210302, rs2241880), IRGM (rs13361189) or NOD2 (rs2066844, rs2066845, rs2066847) SNP variants (data not shown).

As Muise and colleagues point out, the association between NCF4 and CD is not found in all GWAS studies. However, the rs4821544 SNP may define a subgroup of patients that develop Crohn's disease in part as consequence of defective granulocyte ROS production. This may also explain why some studies find impaired ROS production in CD patients, whereas others do not; none of these studies have stratified their patients according to genetic risk factors. Obviously, carrying the rs4821544 can not be the only factor involved in development of CD, as healthy people also bear this

mutation. However, impaired bacterial clearance in patients carrying this risk allele may contribute to the risk of getting CD.

Our results demonstrate for the first time that risk-conferring SNPs within the NADPH oxidase machinery lead to in functional alterations in granulocyte ROS production in CD patients. These data also show that although many of the SNPs found to be linked to CD, including rs4821544, are synonymous, they may nevertheless convey functional consequences.

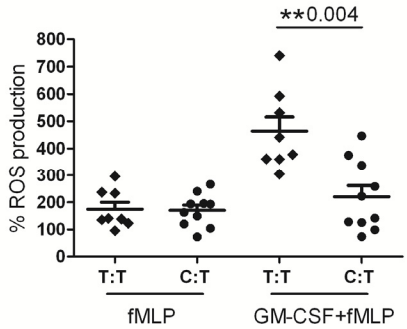

Figure 1: Production of N-formyl-methionyl-leucyl-phenyalanine (fMLP)-stimulated reactive oxygen species (ROS) is decreased in granulocyte-macrophage colony-stinulating factor (GM-CSF)- primed neutrophils from patients with Crohn`s disease bearing the NCF4 risk allele (C:T). Freshly isolated granulocytes were pretreated with 5ng/ml GM-CSF and stimulated with 1μM fMLP for 30 min. Intracellular ROS production was measured by dihydrorhodamine-123 assay as described [4] and the increase in intracellular ROS as a result of stimulation was presented as a percentage of control (unstimulated cells). The mean ±SEM values of C:T heterozygous (n=10) and T:T homozygous (n=8) patients with Crohn`s disease is shown. Mann-Whitney test for unpaired samples was performed. Significant differences were also seen when GM-CSF primed cells were stimulated with fMLP for 15 min (not shown).

	NCF4 variant	Gender	Age at diagnosis	Duration of disease	Disease location	Fistuling disease	Treatment	Other
1	T:T	F	30	1	Colonic	No	Mesalazine, entocort	
2	T:T	M	15	18	Ileocolonic	No	Azathioprine	
3	T:T	M	20	1	Ileocolonic	Yes	Prednison	
4	T:T	M	18	6	Ileocolonic	Yes	Prednison, MTX	Severe small bowel disease
5	T:T	M	20	47	Ileocolonic	Yes	Entocort	
6	T:T	F	15	11	Ileocolonic	Yes	Adalinumab	Severe small bowel disease
7	T:T	M	12	11	Ileocolonic	No	Adalinumab	Pioderma
8	T:T	M	52	11	Ileocolonic	Yes	Infliximab	
9	C:T	M	18	21	Ileocolonic	No	Adalinumab	Thrombosis
10	C:T	F	29	4	Colonic	No	Adalinumab	Sacro-ileitis
11	C:T	F	17	13	Ileocolonic	No	Azathioprine	
12	C:T	M	20	16	Ileal	No	Prednison, MTX	
13	C:T	M	30	17	Ileocolonic	Yes	Adalinumab	Sacro-ileitis
14	C:T	F	23	20	Ileal	No	Adalinumab	
15	C:T	F	33	19	Ileocolonic	Yes		Severe small bowel disease
16	C:T	F	57	4	Ileocolonic	No	Prednison, Adalinumab	
17	C:T	F	16	25	Ileal	Yes	Azathioprine	
18	C:T	M	30	2	Ileocolonic	No	Adalinumab	

Table 1: Patient characteristics

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Chapter 5

Defective GM-CSF signaling in granulocytes from Crohn`s disease patients carrying the NCF4 risk-allele

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In preparation

Chapter 6

Adding fuel to the fire: acquisition of antigen presenting cell characteristics by mucosal granulocytes from patients with Crohn`s disease

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Submitted

Chapter 7

Maintaining spontaneous and pharmacologically induced remission in Crohn`s disease by active suppression of p21Rac1 signalling

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Submitted

Chapter 8

Decreased SHIP1 activity in Crohn`s disease

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Chapter 9

Summary & discussion

Summary and Discussion

The role of phagocytes in IBD

Although inflammatory bowel disease (IBD) is generally accepted to develop as a result of a limited innate immune response followed by a hyperactive adaptive immune response to the commensal luminal bacteria, the exact role of phagocytes in IBD development is still controversial. In this thesis, we have focussed our attention on the contribution of intrinsic defects in phagocyte cell function to IBD pathology. Polymorphonuclear neutrophils (PMN) and monocytes are part of the innate immune system, which forms the first line of defence against invading microbes. They quickly migrate from the circulation to the site of inflammation, phagocytose the pathogens and resolve the inflammation through bacterial killing. Biomedical literature strongly supports the notion that innate immune dysfunction is associated with the pathogenesis of many autoimmune diseases. We therefore set out to investigate the cellular functions of phagocytes in IBD. However, when performing functional studies with the phagocytes, it is imperative to know to what extent primary cell isolation procedures can affect these cell functions. Therefore, i**n chapter II** we performed a systematic evaluation of the most frequently used methodologies for the isolation of PMN and monocytes from peripheral blood, and the influence thereof on phagocyte cell function. This chapter presents the reader with the advantages and disadvantages of the different isolation methods employed in terms of cell purity, yield and functionality. PMN were isolated by Ficoll density gradient, positive selection or Polymorphprep density centrifugation. Although functional cell characteristics scored well in PMN isolated by Polymorphprep, the quality of the isolates was inconsistent. Positive selection yielded the highest purity, but lowest cell numbers, precluding this isolation method for assays requiring large cell numbers. Although Ficoll density gradiation isolates performed slightly worse in ROS production assays, this method is relatively inexpensive and easy. Interestingly, significant differences were observed in cell surface expression of Toll-like Receptors upon different isolation procedures. Although this did not result in differences in *E. Coli* phagocytosis, other assays may potentially be affected, showing the importance of choosing the proper isolation method, and maintaining this method throughout the conducted study. For monocytes, isolation procedures tested included positive selection, negative selection, adherence and Rosettesep. Though costly, positive selection of monocytes showed a remarkably superior purity to other isolation procedures. The overall conclusions drawn here represent our view that if high purity of cells is required and limited numbers of cells are needed, positive isolation of PMN and monocytes are the most suitable methods. For experiments where high purities of phagocytes are not stricktly required and financial restraints play a role, ficoll density gradient centrifugation and the traditional adherence isolation procedure remain good options for PMN and monocyte purification, respectively. Based on these observations, Ficoll density centrifugation for PMN and positive selection of monocytes were the methods of choice for our subsequent studies.

It is clear that congenital neutrophil defects lead to diseases (CGD, LAD) which often present with intestinal inflammation. As described in **Chapter I**, PMN can potentially contribute to mucosal inflammation in a number of ways[1]. In **Chapter III,** we investigated the presence of inherent functional defects in PMN, which may play a role in the development of primary IBD or its extraintestinal manifestations. To this end, we compared signal transduction patterns and corresponding cellular functions of CD PMN with those of healthy controls. We observed that IL-8, which mediates migration of PMN through the activation of the PI3K and ERK1/2 signaling pathways, triggers a normal migratory response in CD PMN, in conjunction with an unaltered IL-8-mediated PKB/AKT and ERK1/2 phosphorylation in these cells. However, in a more physiologically relevant setting, PMN basal-to-apical migration across epithelial cells (Caco2) towards IL-8 was found to be deficient in CD patients. We speculate that enhancement of CD11b expression on PMN by IL-8 may be associated with increased adhesion and reduced migration to the apical region.

Loss of NADPH oxidase activity, leading to reduced bactericidal activity of PMN and defective ROS production in several inherited disorders, is highly associated with intestinal inflammation which is undistinguishable from CD. However, there are several discrepancies in literature regarding NADPH activity in CD. In our cohort, we observed an enhanced fMLP-mediated ROS production, without prior priming with proinflammatory cytokines. This was reflected by an enhanced fMLP-induced PKB and ERK1/2 signaling in these CD patients. Though counterintuitive, this inappropriate respiratory burst may contribute to the epithelial damage observed in CD[2]. ROS are released in the mucosa and are known to contribute to the loosening of cell-cell contacts which normally define a healthy epithelial layer. This in turn results in an enhanced invasion of bacteria thereby triggering an positive feedback loop and inflammatory response.

In this chapter, we also establish the fact that PMN survival signaling is deregulated in CD patients. We demonstrate a decreased caspase 3 and 8 cleavage during spontaneous apoptosis as well as the rescue thereof by GM-CSF, which surprisingly, is not reflected by Annexin V or TUNEL staining. We went on to show that both PKB and STAT3 activation is severly reduced in PMN from CD patients in response to GMCSF stimulation. Thus, a reduced survival signal emanating from this pathway may counteract the enhanced survival mediated through reduced caspase cleavage. On a more general note, these results caution against the common practice of using only one apoptotic marker in the investigation of cell death. In total, we demonstrate that instrinsic defects in PMN transepithelial migration, ROS production, chemokine and cytokine-induced signaling are present in CD patients. Through these and other studies, a modulating role for PMN in the development of CD is becoming ever more obvious.

Genetic IBD risk factors influencing PMN function

Although our studies in Chapter III demonstrate significant differences in some cellular and molecular pathways between CD patients and healthy controls, we observed some variabiliy between individual patients. We entertained the notion that this variability may be due to differences in genetic background, and that by pooling all patients,

defects in PMN function that are present in only a subset of patients may go unnoticed. Stratifying patients according to genetic risk factors may unveil these specific defects. However, the function of most of the IBD risk loci identified to date remains obscure, as is the cell type most likely affected by these single nucleotide polymorphisms (SNPs). Interestingly, however, one clear candidate for investigation presented itself: a T to C substitution in intron 1 of the gene *NCF4* was shown to be associated with CD (rs4821544). This gene encodes the protein $p40^{phox}$, a component of the NADPH oxidase complex, which is essential for PMN ROS production. As PMN are one of the main sources of ROS in the human body, it seems likely that if any cell is going to be affected by this SNP, it would be these phagocytes. In **Chapter IV** we investigated this hypothesis, by comparing PMN ROS production between CD patients carrying the NCF4 risk allele, C, and patients carrying the non-risk T alleles[3]. Interestingly, upon the detection of bacterial peptides, the major burst of ROS produced by PMN primed by the proinflammatory cytokine GM-CSF, was significantly reduced in patients carrying the rs4821544 SNP. This means that, for the first time, CD patients can be stratified based upon their genetic status into efficient ROS producers and poor ROS producers, which may have clinical implications, as a poor ROS production could interfere with bacterial clearance, and thus contribute to the ongoing inflammation. Of note, the use of GM-CSF as an innate immune-boost in the treatment of IBD has long been investigated, but these studies are now largely discontinued as a result of conflicting reports on efficacy. Based on our data, it is possible that GM-CSF responders may be identified based on their NCF4 genetic profile, and it might be worthwhile revisiting this therapeutic avenue.

Theoretically, it is possible that NCF4 C-allele patients have a normal ROS production in their GMCSF-primed PMN, but that ROS in T-allele patients is enhanced. This would be in line with the observation in Chapter III that fMLP-induced ROS production is increased in CD patients compared to healthy controls, whereas ROS production in GM-CSF primed cells is unaffected. Pooling the C and T allele patients would obscure the defect in GM-CSF-primed respiratory burst, whereas no differences were observed in unprimed PMN between C and T-allele patients. Further comparison between CD patients and T- and C-allele carrying healthy controls would be required to formally prove this. However, our results in **Chapter V** argue against this hypothesis. In this chapter, we observe a reduced GM-CSF-induced STAT3 phosphorylation in PMN from patients carrying the C-allele. In conjunction with the reduced STAT3 activation observed in GM-CSF-triggered CD PMN compared to healthy controls in **Chapter III**, this suggests that PMN from C-allele patients are less susceptible to certain stimuli compared to both T-allele patients and healthy controls.

In **Chapter V**, we set out to further identify the mechanism through which an intronic SNP in NCF4 could cause a reduced ROS production in C-allele patients. NADPH oxidase function requires the activity of each of its individual components (i.e. $p91^{phox}$, $p22^{phox}$, $p67^{phox}$, $p47^{phox}$, $p40^{phox}$ and Rac2). In the case of the GTPase Rac, this means the conversion of a GDP-bound form to the GTP-bound form of the protein. As IBD-related SNPs in NCF2 were found to interfere with Rac2 binding and activity, we first investigated the effect of NCF4 rs4821544 on Rac2 activity. However, no differences in

Rac2 GTP loading were observed between C-allele and T-allele carrying patients. Next, we investigated the phosphorylation of the NCF4 product $p40^{phox}$, and observed that in patients carrying the NCF4 risk allele, $p40^{phox}$ phosphorylation is same as the wild type. Hence, it is conceivable that rs4821544 doesnot interferes with protein function. However, other factors appear to be at play; whereas ROS production in GM-CSF primed PMN was severely reduced in C-allele patients, this effect seemed to be restricted to this cytokine as priming with the proinflamatory agent G-CSF elicited a normal ROS response in C-allele patients compared to T-allele carriers. Interestingly, the gene encoding the GM-CSF receptor β subunit, *CSF2RB*, lies adjacent to NCF4. Thus, we postulate that the NCF4 risk allele may confer a defect in GMCSF-receptor signaling. After excluding the possibility that GM-CSF receptor expression itself is decreased in NCF4-risk allele carrying patients, we analysed the signal transduction pathways initiated through this receptor. We observed reduced activation of STAT3, STAT5, PKB, but to a lessed extent ERK1/2, in C-allele PMN stimulated with GM-CSF. The GM-CSF receptor is a heterodimer composed of a ligand binding α-subunit, and a transmembrane signaling β-subunit. This latter is shared between the GM-CSF-, IL-3- and IL-5-receptors, and a defect in CSF2RB signaling should therefore also be observed upon stimulation of cells with these interleukins. Indeed, IL-5 induced a significantly reduced phosphorylation of STAT3 and PKB in PMN from patients carrying the NCF4 risk allele as compared to T-allele carriers. Thus, we provide compelling evidence that the presence of a C-allele of the NCF4 gene confers a CSF2RB signaling defect in CD patients. How an intronic SNP in one gene is able to affect the functioning of its neighbouring gene on a genetic level is currently under investigation.

CD is a heterogeneous disease, where different underlying mechanisms may cause slightly different disease phenotypes. Genetic variability is likely to contribute to the differences of opinion regarding the role of PMN in CD, and it is possible in that some previous studies innate immune defects were underestimated or even obscured by pooling CD patients with different genetic backgrounds. Improved characterisation of risk alleles involved in innate immune processes would allow the identification of subgroups of patients for whom innate immune deficiency plays a role in their pathogenesis.

A previously unsuspected role for PMN in IBD

Aside from their bactericidal function, a novel role for PMN was recently suggested by the *in vitro* observation that PMN were able to acquire characteristics typical of antigen presenting cells (APC). The importance of these findings was underscored by the fact that such redifferentiated PMN were found to be present *in vivo* in patients suffering from inflammatory disorders such as rheumatoid arthritis. Theoretically, such redifferentiating PMN functioning as APC could act as major contributors to an ongoing inflammation, as APCs are known to activate T-cells, and induce their differentiation and proliferation. **In chapter VI** we investigated whether a similar mechanism could be involved in the ongoing inflammation in CD patients. We showed that peripheral blood PMN do not express the APC markers MHC-II, CD80 or CD86, but that mRNA and protein expression of these markers can be induced upon culture of cells with the cytokine coctail GM-CSF, INFγ and IL-4. The intrinsic propensity of

peripheral blood PMN to dedifferentiate in culture was not affected in CD patients. Strikingly, we did observe dedifferentiated PMN in the intestinal mucosa from CD patients. These APC-PMN were most commonly found in mucosa from patients with active disease, but were less common in patients with inactive disease. In healthy controls, massive PMN influx was absent, and the sporadic PMN detected were negative for APC markers. Interestingly, mucosal PMN in patients with active disease co-expressed APC markers, regardless of whether the biopsy was obtained from an inflammed site or an adjacent non-inflamed region. However, a potential explanation for this came from our demonstration that GM-CSF is overexpressed in both non-inflamed and inflamed biopsies from patients with active disease, whereas it is absent in either inactive patients or healthy controls. Thus, the local cytokine profile may not only rescue PMN from apoptosis (one of the functions of GM-CSF) but can also induce a phenotype consistent with that of antigen presenting cells. The subsequent capacity of PMN to take up, process and present bacterial antigens to T cells can add fuel to an already overactivated TH-1 response in CD, which is known to be involved in its pathogenesis.

The notion that non-inflamed mucosa from CD patients with active disease is not similar to that in patients in remission or healthy controls is not a new one. Previous studies have shown that the non-inflamed regions in IBD patients are not similar to those in healthy individuals[4, 5]. Our own study in **Chapter VII** confirms this idea.

p21Rac activity in phagocytes as target for treatment in IBD

One of the great mysteries in IBD pathology, and especially CD, is why mucosal inflammation does not spread to adjacent non-inflamed regions. As mentioned above, these non-inflamed regions are subjected to cytokine patterns and inflammatory responses similar to those in inflamed regions, nevertheless, macroscopically and histologically, no inflammation is present. In other words, what mechanisms keep the uninflamed mucosa from reacting to the proinflammatory signals that are given off by cytokines produced there? In **Chapter VII** we provide some insight into this process. Using kinome profiling we identified a number of signaling pathways that are differentially activated in the inflamed and non-inflammed locations of the same individual as compared to non-IBD controls. Whereas inflamed colon of CD and UC patients showed significant differences in kinase activity patterns, the non-inflamed regions were remarkably similar between these diseases, indicating that a similar anti-inflammatory response may be at play here. The most distinguishing feature observed in non-inflamed biopsies was a two-fold decrease in p21Rac activated kinase (PAK) pathway activity. We provide *in vitro* evidence that this suppression was due to deregulated GAP and GEF activities, resulting in reduced p21Rac activity in non-inflamed colonic mucosa. In contrast, as compared to healthy mucosa, an enhanced activity of this pathway was observed in inflamed biopsies from IBD patients.

Thiopurines (azathioprine, 6-mercaptopurine, 6-thioguanine) are the most effective drugs in CD for maintaining remission, and all act through the common metabolite 6-thioguanine triphosphate (6-TG). Data by Neurath (and Teide et al. 2003) and colleagues[6, 7]show that the end metabolite of thiopurines, 6-TG, specifically

inhibits the activation of p21Rac GTPases, and they provide evidence that the inhibition of p21Rac by thiopurines is, at least partly, responsible for its clinical activity in CD. In our study, we showed that bacterial uptake and killing by monocytes, which is impaired in CD patients as compared to healthy controls, is boosted by treatment of cells with 6TG or selective Rac inhibitors. Thus, reduced p21Rac activity levels contribute to remission in CD patients, and inhibition of p21Rac through therapeutic agents is likely to contribute to maintenance of remission in these patients. It is important to note that the optimal stimulation of innate immune effector function by 6-TG was observed at concentrations which are normally used for treatment of IBD (10-20μM). In this context, thiopurines would serve to help the inflamed site by repressing the activity of the p21Rac GTPase in phagocytes, stimulating their bactericidal activity and thereby inducing remission.

The adaptive immune response in CD

While our studies have shown that a defect in the complex regulatory mechanism regarding the innate immune system is present in IBD, it is clear that there is also a role for the adaptive immune response in IBD pathology. One of the SNPs identified to contribute to risk of IBD development is ATG16L1, a gene involved in autophagy. This gene, which is located in the IBD10 risk locus, plays a role bacterial clearance by innate immune cells. Located directly upstream of ATG16L1 lies the gene *INPP5D*, which encodes for the lipid phosphatase SHIP1. Recent studies[8] have shown that genetic deletion of this phosphatase results in spontaneous development of Crohn`s like ileitis in mice. This phenotype can be rescued by the transfer of SHIP1 competent T-cells, suggesting that colitis in this case is mediated mainly through alteration of the adaptive immune response[8]. Therefore, we measured the SHIP1 expression and activity in peripheral blood mononuclear cells (PBMC) and established that whereas SHIP1 mRNA and protein expression was increased in CD patients compared to healthy controls, the lipid phosphatase activity of the protein was severely impaired. Furthermore, we identified a subpopulation of patients with undetectable or extremely low SHIP1 protein levels, and therefore absent SHIP1 activity. The molecular/genetic reason for the reduced SHIP1 activity in the majority of patients and the loss of SHIP1 protein (despite the presence of SHIP1 mRNA) in others is currently under investigation. We have previously shown that inhibition of SHIP1 activity results in proteasomal degradation of the protein[9]. It is tempting to speculate that a similar process takes place in those patients with reduced SHIP1 expression, although why these and not other patients should be susceptible to this mechanism remains unclear. One possible explanation could be that a compensatory mechanism is in place in the majority of patients, which (over)compensates for the lack of enzymatic activity of SHIP1.

Also under investigation is the exact mechanism through which reduced SHIP1 activity may contribute to ileitis. SHIP1 has been shown to inhibit T-cell apoptosis, suggesting that enhanced T-cell death underlies the colitis in $SHIP^{-/-}$ mice. This is counterintuitive, as reduced susceptibility to T-cell apoptosis has been shown to be one of the mechanisms contributing to on-going inflammation in human CD[10].

However, both the substrate of SHIP1, PtdIns(2,3,4)P_3, and its product PtdIns(3,4)P_2 are required for full activation of PKB and the downstream survival signal[11, 12], and indeed, both SHIP1 agonists and antagonists have been shown to induce cell death[13, 14]. SHIP1 mediated survival is thus not straightforward, and the exact mechanism of T-cell mediated colitis induction in SHIP1$^{-/-}$ mice remains to be elucidated. Of note, enhanced PMN numbers and massive PMN influx is observed in the inflamed mucosa of these mice. A contributory role of the innate immune system in this colitis model can therefore not be excluded.

In light of the decreased SHIP1 activity in CD PBMCs observed in the current study, we propose that, in addition to ATG16L1, SHIP1 may contribute to the risk conferred by 2q37 CD risk locus. This phenomenon is not novel, as it has been observed in chromosome 1, where the risk locus, IBD7 resides. Several genes within the 1p36 locus appear to confer risk for CD (e.g. Runx3, P110δ, RL11), demonstrating that genes with similar biological function map close together on chromosomes and may contribute to disease locus risk. In our studies, SHIP1 expression and activity did not correlate to ATG16L1 risk SNP status of patients, suggesting that these two gene may contribute to disease in an independent manner.

In conclusion

In this thesis, we establish the presence of intrinsic signaling defects in PMN from CD patients, which in turn results in their dysfunction. In addition, we demonstrate a new role for PMN as antigen presenting cells, and demonstrate the presence of these cell in the mucosa from CD patients with active disease. Through these and other studies, a role for the innate immune system in the development of CD is becoming ever more apparent. Exact clarification of the immunological defects may provide targets for the treatment of CD. We provide an exellent example of this by demonstrating that p21Rac inhibition in innate immune cells by 6-TG enhances their phagocytic capacity.

Theoretically IBD is broadly divided in CD and UC, but after numerous investigations over the past years, it has become clear that, biologically speaking, there are at least 3-5 different types of Crohn`s disease. A subgroup of patients suffer from a decrease in paneth cell function (paneth`s disease), some have a defective ROS production (NCF4, NCF2, Rac2) or autophagy (ATG16L1), and in others a defective pathogen associated molecular pattern recognition (NOD2) plays a role in disease. Pooling all CD patients into one group may obscure the role of individual cell types and functions that can play a role in individual patient groups. In future, development of personalised medicine strategies, classifying different subsets of CD patients based on their genetic status and implementing treatment strategies according to their functional cellular deficiencies, may drastically improve disease outcome.

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Nederlandse samenvatting

Nederlandse samenvatting voor niet-ingewijden.

Het immuunsysteem in de darm.

De belangrijkste taak van de darm is om water en voedselstoffen op te nemen uit ons dieet. Het maag-darm kanaal vormt daarbij een essentiële barrière tussen het voedsel dat wij tot ons nemen, en ons lichaam. Het is daarbij van groot belang dat de bacteriën zie zich in het maag-darm kanaal bevinden uit het lichaam geweerd worden. De binnenbekleding van de darm is voorzien van een slijmvlies, wat bestaat uit een epitheel cel laag, beschermd door een slijmlaag die bacteriën vangt en voorkomt dat harde stukjes in ons eten de kwetsbare epitheelcel laag beschadigen. Daarnaast is er nog een tweede beschermingsmechanisme aanwezig; immuun cellen patrouilleren net onder de epitheel cel laag, en sporen bacteriën op die eventueel toch door gedrongen zijn tot de darmwand. Deze patrouillerende immuun cellen behoren tot het aangeboren immuunsysteem, en zijn in staat de bacteriën in zich op te (fagocytose) en te doden. Indien er ergens in deze eerstelijns verdediging iets fout gaat, of de darmwand onverhoopt toch beschadigd raakt, zijn bacteriën in staat door te dringen in het weefsel onder de epitheelcellaag. Er vindt dan een tweeledige immuun reactie plaats. Allereerst worden grote hoeveelheden fagocyten aangetrokken, die de bacteriën opnemen en doden. Vervolgens verschijnt een tweede klasse van aangeboren immuun cellen terplekke; de antigen-presenterende-cellen. Deze cellen nemen geïnfecteerde cellen en bacteriën op, verwerken deze tot kleine eiwitten (antigenen), en presenteren deze vervolgens aan immuun cellen die behoren tot het aangeleerde immuunsysteem. Tot het aangeleerde immuunsysteem behoren B-cellen en T-cellen. B-cellen die de gepresenteerde antigenen herkennen zullen hiertegen antistoffen gaan produceren, terwijl T-cellen die de antigenen herkennen pro-inflammatoire factoren produceren die de afweerreactie verder stimuleren. Als alle bacteriën verwijderd zijn, komt de immuunreactie ten einde.

In sommige gevallen is het immuun systeem niet in staat om een ontstekingsreactie adequaat op te lossen. In dat geval kan er een chronische ontsteking ontstaan en spreken we van een inflammatoire darmziekte.

Inflammatoire darmziekten.

Ongeveer 1 op de 200 mensen leidt aan een chronisch inflammatoire darmziekte (*Inflammatory Bowel Disease*, IBD). Deze ziekten worden gekenmerkt door klachten als buikkrampen, diarree, bloederige ontlasting, verhoogde ontlastingsfrequentie, en gewichtsverlies. Er worden twee typen IBD onderscheiden: Colitis Ulcerosa (CU) en de ziekte van Crohn (*Crohn's disease*, CD). Hoewel deze twee ziektebeelden in sommige opzichten erg op elkaar lijken, zijn er ook belangrijke verschillen. Bij CU is er sprake van een continue ontsteking die zich uitstrekt vanaf de endeldarm. Bij de ziekte van Crohn worden vaak meerdere ontstoken gebieden gevonden, die gelokaliseerd kunnen zijn in ieder deel van het maag-darm kanaal. Deze laesies zijn afgewisseld met normaal, niet ontstoken darm, maar kunnen zich snel uitbreiden en dringen vaak diep in de darmwand door.

Er is geen eenduidige oorzaak aan te wijzen voor het ontstaan van IBD. Algemeen wordt aangenomen dat een verstoorde regulatie van het immuunsysteem

één van de belangrijkste oorzaken is. Ook omgevingsfactoren, zoals roken en voeding spelen een rol. IBD komt op de hele wereld voor, maar vooral in "ontwikkelde" landen in Europa en Amerika. Ook de genetica speelt een belangrijke rol. Hoewel we al langer weten dat kinderen van ouders met IBD een verhoogde kans hebben op het ontwikkelen van CD of UC, worden er pas recentelijk vorderingen geboekt in de identificatie van de genen die hierbij betrokken zouden kunnen zijn. De huidige denkwijze suggereert dat IBD ontstaat door een ongelukkige combinatie van omgevingsfactoren en kleine genetische afwijkingen, die leiden tot een verstoorde immuunreactie in de darm. De huidige medicatie richt zich vooral op het onderdrukken van de darmontsteking, bijvoorbeeld door immuunsuppressiva zoals Azathioprine. De gedachte dat CD gekarakteriseerd wordt door in disbalans in de activiteit van het aangeboren immuunsysteem (te weinig) versus het aangeleerde immuunsysteem (te veel) begint steeds meer geaccepteerd te worden. Deze ontwikkeling wordt beschreven in de inleiding van mijn proefschrift **(hoofdstuk 1)**.

De rol van het aangeboren immuunsysteem in IBD

Een belangrijke speler in het aangeboren immuunsysteem is de neutrofiele granulocyt (kortweg granulocyt). Granulocyten, granulaire (korrelige) cellen, zijn één van de vijf hoofdsoorten leukocyten (witte bloedcellen). Ze vormen de grootste groep leukocyten en maken ongeveer 60% uit van het totale aantal dat in een gezond lichaam aanwezig is. Granulocyten zijn kort levende witte bloedcellen die zowel in het bloed als in ons weefsel voorkomen, en daar de primaire immuunreactie verzorgen om zo infecties snel onder controle te krijgen. Met het werk beschreven in dit proefschrift wilden wij de rol van de aangeboren immuun cellen in het ziekteproces van IBD beter in kaart brengen, met een focus op de invloed van de granulocyten. Om deze cellen te bestuderen dienen ze uit het perifere bloed te worden geïsoleerd, een proces waarbij ongewild activatie van de granulocyten kan plaatsvinden. Om die reden besloten we allereerst de methodologie om deze cellen te kunnen isoleren te optimaliseren.

Hiertoe vergelijken we in **hoofdstuk 2** een aantal verschillende protocollen die veel gebruikt worden voor de isolatie van fagocyten. Verschillende protocollen hebben hun eigen voor en nadelen en beïnvloeden functionele parameters van de geïsoleerde cellen op verschillende wijze. Wij concluderen dat de optimale keuze van het isolatieprotocol afhangt van het type onderzoek dat gedaan gaat worden met de geïsoleerde cellen. Het vervolg onderzoek beschreven in dit proefschrift maakt afhankelijk van de exacte situatie dan ook gebruik van verschillende protocollen.

In **hoofdstuk 3** worden de functionele eigenschappen van het neutrofielencompartiment in CD bestudeerd. Er ontstaat een complex beeld van verschillende eigenschappen van de granulocyt in de ziekte van Crohn. Zo zien we dat op biochemisch niveau dat de reactie van de granulocyten op de pro-inflammatoire groeifactor interleukine 8 niet wezenlijk verschillend is wanneer CD patiënten worden vergeleken met controles, maar dat in een meer complexere (levensechte?) analyse, waarbij granulocyten door een epitheellaag heen moeten migreren, de granulocyten uit de CD patiënten gemankeerd waren. Dit zou kunnen betekenen dat granulocyten van CD patiënten minder goed in staat zijn om de plaats van bacteriële infectie te bereiken. Omgekeerd zagen we dat fMLP (een bacterieel peptide) juist verhoogde

signaal transductie gaf, een fenomeen dat gepaard ging met een versterkte productie van zuurstofradicalen. Deze zuurstofradicalen dragen in belangrijke mate bij aan de potentie van granulocyten om bacteriën te doden. Echter, een overmaat aan radicaalproductie kan ook leiden tot epitheellaag schade, en kan dus een negatieve rol spelen in IBD. Tenslotte zagen we dat de granulocyten van patiënten met CD verminderde trofische signalering in hun intracellulaire biochemie hadden, wat zou kunnen leiden tot een verminderde overleving van deze cellen. Dit effect wordt echter gecompenseerd door een corresponderende verlaagde pro-apoptotische signalering, waarbij duidelijk wordt dat op biochemisch niveau een disregulatie van signalering plaats vind bij patiënten met CD. Ofschoon deze experimenten dus geen eenduidig beeld opleverden, laten ze wel zien dat het granulocyten compartiment op belangrijke wijze wordt beïnvloed in CD.

De rol van genetische risicofactoren in CD.

Meer duidelijkheid brachten experimenten waarin patiënten eerst gestratificeerd werden op genetische defecten en toen onderzocht werden op de functionele eigenschappen in het granulocytencompartiment. Momenteel zijn meer dan 160 verschillende genetische risico factoren geïdentificeerd, die allemaal een klein deel van het risico op het krijgen van de ziekte van Crohn dragen. Van vele van deze genen wordt gespeculeerd dat ze betrokken zijn in de regulatie van het aangeboren immuunsysteem, hoewel er nog relatief weinig experimenteel bewijs voor is. Eén van de risicofactoren voor het ontwikkelen van CD is een genetische variatie in het gen *NCF4*. Dit gen speelt een belangrijke rol in de productie van zuurstofradicalen, aangezien het codeert voor het eiwit $p40^{phox}$, een component van het NADPH oxidase complex, verantwoordelijk voor deze radicaal productie. In **hoofdstuk 4** laten we zien dat granulocyten van patiënten die het zogenaamde *NCF4* risicoallel dragen gemankeerd zijn in de productie van zuurstofradicalen. Belangrijk hierbij is op te merken dat niet zo zeer de basale zuurstofradicaalproductie aangedaan was, maar met name de productie van deze radicalen na een voorgaande stimulatie met GM-CSF. Dit is een fysiologisch relevant mechanisme; pro-inflammatoire groeifactoren zetten de granulocyten als het ware op scherp en veranderen ze in zuurstofradicaalbommen die afgaan op het moment dat de cel in contact komt met bacteriële peptiden. Dit proces voorkomt dat een granulocyt willekeurig zijn zuurstofradicalen loslaat (en hiermee mogelijk schade veroorzaakt aan het omliggende weefsel), en alleen in inflammatoire setting grote hoeveelheden radicalen gaat aanmaken. Dit onderzoek toont dus aan dat een kleine genetische afwijking in een gen kan leiden tot een verminderde functionaliteit bij patiënten met de ziekte van Crohn. Dit was een belangrijke observatie, omdat voordat wij deze waarneming deden er nog geen correlaties in de literatuur beschreven waren tussen genotype en fenotype m.b.t. CD.

In **hoofdstuk 5** wordt dieper ingegaan op de moleculair mechanistische achtergrond die ten grondslag ligt aan onze bevinding. In de studies beschreven in dit hoofdstuk elimineren wij een groot aantal mogelijke verklaringen en laat ik tegelijkertijd zien dat het verschil in zuurstof radicaal productie tussen *NCF4* risicoallel-dragende en niet-dragende patiënten terug te voeren is tot alternatieve activatie van één van de ketens van de GM-CSF receptor. In dit hoofdstuk tonen wij aan dat zuurstof

radicaal productie in granulocyten van CD patiënten met het *NCF4* risicoallel alleen verminderd is wanneer de cellen met GM-CSF in verhoogde staat van paraatheid worden gebracht, terwijl andere pro-inflammatoire groeifactoren een normale respons bewerkstelligen. Hoewel er voldoende GM-CSF receptoren op de oppervlakte van de cel aanwezig zijn, is de biochemische activatie van intracellulaire signalering desalniettemin verminderd in patiënten die drager zijn van het *NCF4* risicoallel. De GM-CSF-receptorketen wordt gedeeld met de receptor voor IL-5, en ook activatie van signalering met dit cytokine is verstoord in deze patiënten, wat er sterk voor pleit dat het dragen van het risicoallel voor *NCF4* een defect in deze receptorketen met zich mee brengt. Hoe het *NCF4* risicoallel precies gerelateerd is aan deze alternatieve receptorketen activatie blijft vooralsnog echter onduidelijk, maar het is intrigerend om te constateren dat het NCF4 risicoallel genomisch vlak naast deze GM-CSF receptor keten in het DNA ligt.

Een nieuwe functie van granulocyten in IBD

Ofschoon in de klassieke interpretatie van de immunologie niet gesproken wordt over een communicatie tussen granulocyten en het aangeleerde immuunsysteem, zijn er de laatste tijd aanwijzingen in de literatuur dat deze cellen onder bepaalde omstandigheden eiwitten aan het aangeleerde immuunsysteem kunnen gaan presenteren, een taak die normaliter alleen is voorbehouden voor specialistische antigen presenterende cellen (APCs). Theoretisch zou een dergelijk proces een belangrijke rol kunnen spelen in het aanwakkeren en brandend houden van een ontstekingsreactie, iets wat precies het probleem is in CD. Inderdaad troffen wij in het werk beschreven in **hoofdstuk 6** in de mucosa van CD patiënten granulocyten aan die karakteristieken hadden van APCs en blijkbaar antigen aan het presenteren waren aan het aangeleerde immuunsysteem, terwijl dit eigenlijk niet geval was in de mucosa van gezonde individuen. *In vitro* analyses toonden aan dat granulocyten met deze APC kenmerken inderdaad in staat zijn T-cellen te activeren en aan te zetten tot celdeling. De reden dat deze cellen gevonden worden in de darmwand van CD patiënten maar niet bij gezonde personen verbonden wij aan het feit dat GM-CSF, één van de groeifactoren die kunnen bijdragen tot deze herprogrammering van granulocyten richting APCs, in verhoogde mate aanwezig zijn in de mucosa van CD patiënten ten opzichte van controles. Verder onderzoek moet nu aantonen hoe belangrijk deze granulocyt-afhankelijke antigenpresentatie is voor het in stand houden van chronische darmontstekingen in IBD.

Beïnvloeding van aangeboren immuunsysteem middels therapie

Het voorlaatste experimentele **hoofdstuk (nummer 7)** richt zich op de verschillen tussen ontstoken en niet-ontstoken gedeelten van de darm in de patiënten met de ziekte van Crohn. Met behulp van geavanceerde kinome profiling technieken worden de verschillen tussen deze twee condities met grote nauwkeurigheid in kaart gebracht. Het blijkt dat met name het eiwit p21Rac een belangrijke speler is in de verschillen tussen ontstoken en niet-ontstoken darm. De niet ontstoken darm wordt gekenmerkt door een matige remming van dit eiwit. Nu zien we ook dat een dergelijke matige remming van p21Rac gepaard gaat met het aanjagen van de aangeboren afweer

middels verbeterde fagocytaire en bacteriotoxische functionaliteit. Thiopurines, die in de kliniek geldt als één van de betere middelen met betrekking tot in stand houden van remissie van ziekte is ook in staat om p21Rac te remmen, en hiermee de functionaliteit van fagocyten te verbeteren. Deze resultaten dragen verder bij aan het beeld dat met name disregulatie van de aangeboren afweer in haar algemeenheid, en die van neutrofielen compartiment in het bijzonder, het primaire immunologische defect vormt in de ziekte van Crohn en dat rationele therapie zich op dit celtype zou moeten concentreren.

Het aangeleerde afweersysteem in IBD

Recent onderzoek heeft aangetoond dat deletie van het gen *INPP5D* in muizen resulteert in het ontstaan van een Crohnse ontstekingsreactie in deze muizen. Dit gen codeert voor het eiwit SHIP1, dat een belangrijke rol speelt in het overleven van cellen. Het feit dat GM-CSF niveaus, alsmede de granulocyt infiltratie in de mucosa verhoogd zijn in de darmen van deze muizen zou pleiten voor een rol voor granulocyt disfunctie in deze dieren. Echter, het toedienen van normale T-cellen geeft verlichting van de ziekte in deze dieren, wat suggereert dat er ook een rol is voor dit gen in de regulatie van het aangeleerde afweersysteem in CD. Om deze hypothese te testen hebben wij in **hoofdstuk 8** de expressie en activatie van het SHIP1 enzym gemeten in perifere bloed T-cellen van patiënten met de ziekte van Crohn, en die vergeleken met gezonde controles. Wij toonden aan dat SHIP1 weliswaar verhoogd tot expressie komt in T-cellen van CD patiënten, maar dat de enzymatische activiteit van het eiwit verminderd is. Tevens vonden wij een kleine populatie van patiënten waarbij het eiwit niet of nauwelijks aanwezig was. Deze data suggereren dat een verlaagd eiwit niveau of mate van activatie van het SHIP1 eiwit in T-cellen ook in humane IBD een rol zou kunnen spelen. Het exacte mechanisme waarop dit enzym de T-cel functionaliteit in CD patiënten moduleert zal nog verder onderzocht worden.

Curriculum Vitae & PhD Portfolio

Curriculum vitae of Rajesh Somasundaram:

Rajesh Somasundaram was born in Chennai (Madras), India, on the 19th of March 1984. He did his schooling at Vellayan Chetiyar Higher Secondary School from 1996 till 2002. He obtained his Bachelors degree in Plant Biology & Plant Biotechnology from the University of Madras in 2005, followed by an M.Sc in Biotechnology from the University of Madras, India 2007. Early 2008 he moved to the Netherlands to pursue his research career in the Netherlands through an Ubbo Emmius Scholarship at the University Medical Centre Groningen, where he did an intership until May 2010 under the supervison of Prof. dr. Frans Kroese and Prof. dr. Nico Bos. He did two projects: 1) Local expansion of IgA plasma cells in mouse small intestine and 2) $sIgD^+$ plasma cells in human tonsils. From June 2010 he started working as a PhD student under the supervision of Prof. dr. Maikel Peppelenbosch, Dr. Gwenny Fuhler and Dr. Janneke Van der Woude on a project entitled: ``*Action and function of Neutrophils in Crohn`s disease"* and he will defend his PhD thesis on June 19th 2013.

PhD Portfolio

Name: Rajesh Somasundaram
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PhD period: June 2010- June 2013
Promotor: Prof. Dr. Maikel P. Peppelenbosch
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Certificates:
Research management for PhD-students- 03 March & 7 April 2011 (1.0ECTS)
Basis introduction course on SPSS 12-14 January 2011 (0.6 ECTS)
Workshop Browsing genes and genomes with UCSC-27 May 2011(0.4ECTS)
Workshop Photoshop and Illustrator CS5- 27 September 2011 (0.3ECTS)
Workshop Indesign CS5, 28th September 2011 (0.3ECTS)
Course on SNPs and human dieases VIII - November 21-25, 2011 (2.0ECTS)
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Writing biomedical English 8, 22 December 2011 & 15, 29 March 2012 (2.0 ECTS)
Design and data analysis of microarray expression studies, 6-17 October 2008, UMCG (2.0 ECTS)
Genetic epidemological research and data analysis, 22-24 April 2009, UMCG (1.0 ECTS)
Mobitech summer course- Techniques in molecular biology, May-June 2008, UMCG (4.0 ECTS)
Course on bioinformatics- April 7-11, 2008 –UMCG (1.0 ECTS)
GCP/GLP, May 11-20, 2009 (UMCG, 3.0 ECTS)

Student supervision:

- BSc student, Mr. Marvin Da Silva education: biomedical researcher, duration: 4months (February –May, 2011), project title: *Defective GM-CSF signaling in granulocytes from Crohn's disease patientscarrying the NCF4 risk-allele.*
- MSc student, Mr. Eelke Brandsma, education: Biology, duration: 6months (January –June, 2010), project title: *Evidence for secondwave of expansion of B cells in lamina propria of mouse small intestine.*
- BSc student, Ms. Annemarie, education: biomedical researcher, duration: 3months (April –June, 2010), project title: *Characterisation of sIgD plasma cells in human tonsils.*

- MSc student, Ms. Joke Drijvers, education: Biomedical researcher, duration: 5months (March–October, 2008), project title: *Role of notch signaling during differentiation of mature B lymphocytes.*

Attended scientific conferences:

- DDW 2013, May 18-21, 2013 Orlando, Flordia.
- European Crohn`s and Colitis conference (ECCO), 2013, Vienna, Austria
- ECCO 2012, Barcelona, Spain
- ECCO 2011, Dublin, Ireland
- Dutch gastroenterology society meeting (NVGE), The Netherlands (2011 &2012).
- Dutch Immunology society (NVVI), The Netherlands (2008 &2009).
- Young Initiative Crohn and Colitis (Y-ICC), the Netherlands, 2011&2012.

Poster presentations:

- *Adding fuel to fire- Neutrophils as antigen presenting cells in Crohn`s disease*, European Crohn`s and Colitis Organisation (ECCO) 2013, Vienna, Austria.
- *Defective GM-CSF signaling in granulocytes from Crohn's disease patients carrying the NCF4 risk-allele*, ECCO 2013, Vienna, Austria.
- *Defective GM-CSF signaling in granulocytes from Crohn's disease patients carrying the NCF4 risk-allele,* MolMed day, Erasmus Medical Center Rotterdam, 2013.
- *Decreased neutrophil functions and signaling in Crohn`s patients*, ECCO 2012, Barcelona, Spain
- *Decreased neutrophil functions and signaling in Crohn`s patients*, ECCO 2012, Dublin, Ireland.
- *Decreased neutrophil functions and signaling in Crohn`s patients*, NVGE 2012.
- *Characterisation of sIgD plasma cells in human tonsils, NVVI 2009.*

Oral presentations:

- *Defective GM-CSF signaling in granulocytes from Crohn's disease patients carrying the NCF4 risk-allele*, Digestive Disease Week 2013 (Due to present)
- *Defective GM-CSF signaling in granulocytes from Crohn's disease patients carrying the NCF4 risk-allele*, presented at NVGE 2013
- *Adding fuel to fire- Neutrophils as antigen presenting cells in Crohn`s disease*, presented at NVGE 2013.
  Adding fuel to fire- Neutrophils as antigen presenting cells in Crohn`s disease, MolMed Day, Erasmus medical center 2013.

Grants:
Travel grant from NVGE (to attend DDW 2013).
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List of Publications

Somasundaram R* Zhou L*, , Nederhof RF, Dijkstra G, Faber KN, Peppelenbosch MP, Fuhler GM. *Impact of human granulocyte and monocyte isolation procedures on functional studies.* Clin Vaccine Immunol. 2012 Jul;19(7):1065-74. (*equal contribution)

Yuvaraj S, Al-Lahham SH, **Somasundaram R**, Figaroa PA, Peppelenbosch MP, Bos NA. *E. coli-Produced BMP-2 as a Chemopreventive Strategy for Colon Cancer: A Proof-of-Concept Study.* Gastroenterol Res Pract. 2012;2012:895462. Epub 2012 Jan 19.

Somasundaram R*, Deuring JJ, van der Woude CJ, Peppelenbosch MP, Fuhler GM. *Linking risk conferring mutations in NCF4 to functional consequences in Crohn's disease.* Gut. 2011 Oct 24.

Somasundaram R Regeling A, , de Haar C, van der Woude CJ, Braat H, Peppelenbosch MP. *Role of defective autophagia and the intestinal flora in Crohn disease.* Self Nonself. 2010 Oct;1(4):323-327. (*equal contribution)

Fuhler GM, Baanstra M, Chesik D, **Somasundaram R**, Seckinger A, Hose D, Peppelenbosch MP, Bos NA. *Bone marrow stromal cell interaction reduces syndecan-1 expression and induces kinomic changes in myeloma cells.* Exp Cell Res. 2010 Jul 1;316(11):1816-28. Epub 2010 Mar 20.

Hendricks J, Terpstra P, Dammers PM, **Somasundaram R**, Visser A, Stoel M, Bos NA, Kroese FG. *Organization of the variable region of the immunoglobulin heavy-chain gene locus of the rat. Immunogenetics.* 2010 Jul;62(7):479-86. Epub 2010 May 5.

Somasundaram R, Deuring JJ, van der Woude CJ, Peppelenbosch MP, Fuhler GM. *Altered cell signalling and neutrophil functions in Crohn`s disease patients with skin manifestions.* (Submitted)

Somasundaram R , Sandra Fernandes, Johannes J. Deuring, Colin de Haar, Lauran Vogelaar, C. Janneke van der Woude, Maikel P. Peppelenbosch, William G. Kerr, Gwenny M. Fuhler, *Decreased ship-1 activity in Crohn`s disease.* (In preparation)

Somasundaram R, Deuring JJ, van der Woude CJ, Peppelenbosch MP, Fuhler GM. *Adding fuel to the fire: acquisition of antigen presenting cell characteristics by mucosal granulocytes from patients with Crohn's disease.* (Submitted)

Somasundaram R, Deuring JJ, van der Woude CJ, Peppelenbosch MP,Fuhler GM. *Defective CSF2RB signalling in Crohn`s disease patients bearing the NCF4 risk allele.* (In preparation).

Somasundaram R*, Kaushal Parikh*, Lu Zhou*, Gwenny M. Fuhler, Jasper Deuring, Tjasso Blokzijl, Anouk Regeling, Ernst J. Kuipers, Rinse Weersma, Veerle Nuij, Maria Alves, Lauran Vogelaar, Lydia Visser, Colin de Haar, Kausilia K. Krishnadath, C. Janneke van der Woude, Gerard Dijkstra,, Klaas Nico Faber, & Maikel P. Peppelenbosch.

Maintaining spontaneous and pharmacologically-induced remission in Crohn`s disease by active suppression of p21Rac1 signaling. (* equal contribution)
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