Inflammatory bowel diseases
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Chapter 7

Summary, concluding remarks and future perspectives
Summary and concluding remarks
Inflammatory bowel disease (IBD), like other autoimmune diseases, mainly affects young people at the peak of their social and economical productive life and, in the absence of effective therapies, disease symptoms persist for the rest of their life’s. Although current drugs/therapies are usually effective in inducing remission of the disease, symptoms are bound to reoccur at a certain moment. In any case, patients are life-long dependent on drug therapy. It is well-known that prolonged exposure to corticosteroids or immunomodulators is associated with severe complications, including Cushing’s syndrome and cancer. The important task of clinicians and basic scientists now is to find ways to prevent or cure the disease. Thus, in-depth studies have to be undertaken to identify the molecular and cellular mechanisms that lead to the development of Crohn’s disease (CD) and/or Ulcerative colitis (UC). Moreover, detailed studies on the action of currently used drugs may help to further understand disease mechanism and identify novel therapeutic targets (1).

In this study, we undertook genetic approaches to narrow down the search for causative mutations to identify genes that predispose for UC in a Dutch cohort, as well as in a Chinese cohort, where the incidence of this disease is still relatively low. In addition, we studied the molecular and cellular effects of 6-thioguanine (6-TG), the active metabolite of Azathioprine (AZA), in modulating innate immune functions of human monocytes.

RUNX3 and its epistatic gene-gene interactions in UC
RUNX3 is a member of the runt domain family of transcription factors, which are increasingly being recognized to be involved in autoimmunity (2,3). Several observations make RUNX3 a likely candidate gene for IBD susceptibility. First, loss of Runx3 function leads to spontaneous colitis in mice (4). Second, Runx3 is part of the TGF-β signaling pathway, which is considered to be an important immunosuppressive hormone involved in a variety of autoimmune diseases, including IBD (5). More recently it is established that TGF-β is promoting regulatory T cell (Treg) development in the gut. Consequently, impaired RUNX3 activity may result in decreased TGF-β signaling and thus uncontrolled suppression of inflammation leading to intestinal inflammation and IBD (4, 6). Third, the gene encoding for RUNX3 resides on chromosome 1p36, which is a susceptibility locus for IBD (7). From a different angle but similarly important for IBD, mutations in the CD-associated genes SLC22A4/5, encoding the organic cation transporters 1 and 2 (OCTN1/2), were found to disrupt the DNA binding site of RUNX in the promoter element of OCTN1 (8). Thus, we studied the association of RUNX3 in a Dutch cohort of IBD patients and analyzed the putative interaction with SLC22A4/5. We found a significant association of RUNX3-SNP rs2236851 with UC (OR 1.61; 95% CI 1.11-2.32, p=0.02) and this tag-SNP was further associated with pancolitis.

For SLC22A4/5, homozygosity for SNPs rs272893 and rs273900 was significantly associated with CD (OR 2.16; 95% CI 1.21-3.59; p=0.008 and OR 2.40; 95% CI 1.43-4.05; p=0.004, respectively), but not with UC. Both SNPs were associated with an age of onset >40 yrs, ileocolonic localization, non-stricturing non-penetrating behavior and extra-intestinal
manifestations. An epistatic effect of RUNX3 and SLC22A4 was observed for susceptibility for UC (OR 3.83; 95% CI 1.26-11.67, p=0.018).

We analyzed RUNX3 and OCTN1 mRNA expression in inflamed and non-inflamed ileal and colonic mucosa tissue samples from 30 IBD patients (16 CD and 14 UC) and 6 controls. In controls RUNX3 mRNA expression is evenly distributed throughout the colon and the ileum. OCTN1 expression is higher in the ileum compared to the colon (p<0.00001), while the expression level is constant in the different parts of the colon. RUNX3 expression levels are increased in inflamed colonic mucosa compared to non-inflamed colonic mucosa in UC patients. OCTN1 expression is decreased in inflamed colonic mucosa compared to non-inflamed colonic mucosa, which supports the notion that RUNX3 is an inhibitor of OCTN1 expression.

We conclude that polymorphisms in the genomic RUNX3 locus are associated with UC. Moreover, genetic association for CD with SLC22A4/5 was confirmed. Our data, combined with the increasingly recognized role of RUNX3 in autoimmunity, suggest a role for RUNX3 in UC susceptibility and thus provide the first evidence that deregulation of TGF- signaling may play a role in pathogenesis of IBD. This finding warrants further study of the role of this important immunosuppressive pathway in IBD.

The more recent GWA studies did not identify RUNX3 as an evident UC risk gene, although it has been proven to be associated with celiac disease, another intestinal inflammatory disease, at genome-wide level (9). Within the international IBD genetics consortium a meta-analysis has been performed including all UC GWAS data in Caucasians (www.ibdgenetics.org). We have analyzed these data but did not observe compelling evidence for association in the RUNX3 region. However, is should be stressed that it is estimated that only 16% of UC heritability is explained by the 49 confirmed UC loci in this meta-analysis (Anderson et al., submitted) Thus, many additional genomic loci contribute to UC that may be ethnic-, community- and/or environment-specific. Such variants loose their statistical significance when analyzed in a world-wide cohort of UC patients and controls.

Many genes previously identified by candidate gene studies (e.g. Myosin9b, TLR4 and MDR1) have not been confirmed in these GWAS meta-analyses. There are different explanations for these findings. Some genomic regions are poorly covered in GWAS and are therefore not picked up. Some regions might not have reached the stringent statistical threshold applied in GWAS and have therefore not been followed up. Furthermore, there can still be rare variants in these loci that account for part of the “missing heritability”. It is therefore too soon to qualify these genes as false positive findings, especially for those that have been shown to lead to intestinal inflammation when their function is compromised in in vivo models, as for the Runx3 and Mdr1 knockout mice.

To address these questions many of these loci have been included in the Immunochip, which is a custom-made GWAS chip, which is currently being tested in over 25,000 IBD cases and 20,000 controls within the international IBD genetics consortium.
Haplotype-based study to identify UC associated genes in Han Chinese

The incidence UC varies strongly between different ethnicities, especially between the Caucasian and eastern Asian populations (10, 11). The cause of ethnic differences in UC incidence remains unclear, but a distinct genetic background may play a role. There are currently approximately 18 confirmed genes or loci associated with UC in Caucasian patients, the majority having been identified since the introduction of genome-wide association studies (GWAS) during the past few years (12-18). Very little is known whether the same genes also predispose for UC in the Asian population or, alternatively, whether the genetic background of UC in Asia is significantly different from Caucasians. We therefore performed a haplotype-based analysis of six known UC susceptibility loci in Han Chinese UC patients, including \textit{IL10}, \textit{IL2/IL21}, \textit{MYO9B}, \textit{ECM1}, \textit{MST1} and \textit{IL23R} (19).

By analyzing the HapMap database for Han Chinese, we first found that most SNPs associated with UC in Caucasian patients are strongly underrepresented or even monomorphic in the Han Chinese population. Instead of performing a direct replication for the reported SNPs of the above genes, we therefore selected tag SNPs that captured all the major haplotypes covering these genetic regions in the Han Chinese population. Secondly, we found that the different linkage disequilibrium (LD) structure of gene regions between the two ethnicities may help to narrow the search for causative mutations. For instance, the \textit{IL2/IL21} locus in Caucasians is in complete LD, while \textit{IL2} and \textit{IL21} reside in two independent LD blocks in Han Chinese. Our study included 245 UC patients and 300 healthy controls. Though this is a small cohort in comparison to the recent GWASs, thus far this is one of the largest UC cohorts reported from China. Importantly, with this limited sample size, we were able to identify strong association signals in both the \textit{IL2} and \textit{IL21} containing genomic regions, implying that both genes are genetic risk factors for UC. Thus, this study on UC in Han Chinese clearly increases our understanding of the \textit{IL2/IL21} gene locus, where an independent contribution of \textit{IL2} and \textit{IL21} to UC can not be established in genetic association studies in the Caucasian population. It still needs to be determined whether both genes are indeed also associated with UC in Caucasians for which large scale studies with dense finemapping arrays are underway. In addition to the \textit{IL2/IL21} locus, moderate genotype or subphenotype associations were found for \textit{MYO9B} and \textit{IL23R} in UC patients of Han Chinese descent. Larger cohorts of Chinese UC patients are needed to firmly establish these associations. Our study is the first systematically-designed haplotype-based cross-ethnicity UC genetic replication study and points out the importance of the HapMap project for such studies. Moreover, our data emphasize the partly shared genetic background between populations with high and low incidence of UC.

Azathioprine increases human monocyte innate immunity

Accumulating evidence suggests that the primary immune defect in patients with CD lies in impaired innate immunity, specifically in impaired control of the intestinal bacteria and production of inflammatory cytokines. The weak immune response on the one hand predisposes for the accumulation of intestinal bacteria to breach the mucosal barrier and on
the other hand may cause an exaggerated chronic lymphocyte immune response (adaptive immunity) via disordered antigen presentation (20, 21).

For decades, immunosuppressive agents, such as Azathioprine (AZA), have been the first line therapy for treating CD patients. The active derivatives 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG) were shown to suppress adaptive immunity through the induction of apoptosis in the lymphocyte compartment (21). Since recent data suggest that biological pathways important for innate immune functions are impaired in CD, it is relevant to determine whether current drugs also affect innate immunity. Retrospectively, it may turn out that this is actually the prime therapeutic effect of these drugs. Unraveling this mechanism will obviously improve our knowledge of CD therapy.

In our study, we found that 6-TG directly enhances bacterial phagocytosis by human peripheral monocytes. Moreover, it induced the IL-8 production of these cells. Both features are important for the innate immune response. 6-TG-stimulated innate immunity appeared to be mediated through inhibition of the small GTPase Rac1, a previously identified target of this drug in T-lymphocytes. Remarkably, Rac1 inhibition leads to apoptosis in T-lymphocytes and is thus regarded as an immunosuppressant (21). In contrast, Rac1 inhibition leads to increased bacterial phagocytosis by monocytes and in this context can be viewed as an immunostimulant (chapter 6). Importantly, the Rac1 inhibitory effect of 6-TG appeared to be much more effective in monocytes compared to lymphocytes, suggesting that the primary effect of 6-TG is targeting the monocytes. Interestingly, we observed that Rac1 is superactivated in both peripheral monocytes as well as in the intestinal mucosa of IBD patients and thus may impair the innate immune function of monocytes and monocyte-derived macrophages. We therefore propose that the defect in innate immunity in CD originates from Rac1 hyperactivation and that AZA combats the primary defect in CD by counteracting Rac1.

6-TG enhances the capacity of monocytes to clear bacteria that penetrate into the mucosal layer, which on the one hand directly prevents the development of new lesions in the gut mucosa, and on the other hand may help to repress the exaggerated chronic lymphocyte-mediated inflammation. This order of events is in line with the long-term remission maintenance effect of AZA. Our findings not only provide important support for the notion that CD is primarily a systemic monocyte/macrophage immunodeficiency, but also provide a credible mechanism of action of AZA, which is probably the most widely used medication for combating fulminant autoimmune diseases.

A main issue here is that a certain level of Rac1 activity is essential for efficient pathogen phagocytosis (22) and thus that increased pathogen phagocytosis following AZA-mediated Rac1 inhibition seems counterintuitive. The data presented in chapter 6 do not readily provide a mechanistic explanation as to how the inhibition of Rac1 enhances innate effector functions, including stimulation of phagocytosis and IL-8 production. However, supportive data is available that show that tightly controlled Rac1 activity (not too much, not too little \(\rightarrow\) a Goldilocks-like analogy can be drawn) may be required for efficient bacterial engulfment. This is not an entirely new concept, since it has already been shown that engulfment of
apoptotic cells by macrophages occurs at the lamellipodia where active Rac1 is required, but that down-regulation of Rac1 is required for closure of the phagocytic cup. When Rac1 is constitutively overactivated, as in Rac1-V12, the disassembly of the actin patch and the closure of phagocytic cups are significantly delayed and the ability of macrophages to engulf apoptotic cells is strongly reduced (23). Similarly, constitutive-active Rac1-V12 disrupts bacterial phagocytosis and suppresses receptor endocytosis (24). Moreover, it has been shown that balanced Rac1 activity controls the stabilization of growth cone lamellipodial and filopodial protrusions during neurite outgrowth, and controls filopodia formation, phagocytosis, endocytosis and cell motility in Dictyostelium discoideum. The formation of lamellipodia and filopodia requires the cycling of the Rac1-GTPase between the active (GTP-bound) and inactive (GDP-bound) forms, which is catalyzed by Dbl family guanine nucleotide exchange factors (GEFs). The Rac1 inhibitor NSC23766 (which was used in this thesis to provide independent confirmation that 6-TG acts through Rac1) significantly inhibits the binding of Rac1-specific GEFs including Trio and Tiam1 to Rac1, which in turn dose-dependently reduces Rac1-GTP level and the lamellipodia formation under stimulation (25). Therefore, it is plausible to predict that 6-TG sustains Rac1 activity of monocytes within a proper dynamic range, which facilitates phagocytosis. In the context of the inflamed intestine, 6-TG may rebalance Rac1 activity to a level that allows efficient bacterial phagocytosis (a Goldilocks-like “just-right” level of Rac1 activation). As for the mechanism by which 6-TG increases IL-8 production, it has been shown that Rac1 regulates NADPH oxidase-dependent (Nox-dependent) superoxide (O2•−) production by binding to superoxide dismutase-1 (SOD1) (26). In human neutrophils, inhibition of NADPH oxidase increases IL-8 mRNA level and IL-8 production (27). Thus, it is tempting to speculate that 6-TG increases IL-8 production via inhibition of the Rac1-NADPH oxidase pathway, and experiments assessing the effects of Nox inhibitors on the IL-8 production by monocytes should be initiated to provide further support for this notion.

Additional actions of 6-TG should, however, also be considered in relation to its therapeutic effect in Crohn’s disease. The recent GWA studies and subsequent mechanistic studies have clearly implicated a role for autophagy in the pathogenesis of the Crohn’s disease (see for reviews 28, 29). Autophagy is classically considered a cell survival pathway in which cells recycle cellular constituents in conditions of nutrient limitation. Nowadays it is clear that there is an intimate relationship between the molecular mechanisms that control autophagy and the degradation of intracellular pathogens after being phagocytosed. Impaired autophagy may thus lead to compromised clearance of intestinal microbes, which in turn may initiate an inflammatory reaction. 6-TG has been shown to induce autophagy in cancer cells (30-32). It is therefore highly relevant to determine whether enhancement of autophagy is also part of the therapeutic effect of 6-TG in IBD.

Future perspectives
Implications of GWAS data for analyzing the role of monocytes in IBD

The improvement of our knowledge on the genetic profile that predisposes to IBD has revealed intriguing perspectives for biologists to uncover the pathogenesis of the disease. The next step is to unravel the function of the IBD susceptibility genes and how their malfunction leads to disease symptoms. Together with the rapid progress of cost-effective full genome sequencing that makes it possible for individuals to have their entire genome sequenced, personalized treatment of CD and UC patients may become reality within 1 or 2 decades. The socio-economic burden of IBD may be decreased both by reducing the incidence of the disease by personalized prevention as well as personalized therapy for patients.

CD patients have traditionally been considered to have a hyperactive adaptive immune response to environmental antigens. Data from this thesis (chapter 6), as well as recent findings by others (20,33-35), suggest that diminished monocyte function might be the primary immune defect in CD, which in turn drives the exaggerated lymphocyte response. Furthermore, our findings that 6-TG stimulates monocyte phagocytosis and IL-8 production fundamentally challenge the traditional immunosuppressive concept for the treatment of CD patients. To clarify the underlying mechanism of our novel observations, susceptibility genes of CD patients provide us important clues for future studies.

*NOD2*, *ATG16L*, *IRGM* and *IL23R* are the strongest risk loci associated with CD (36-40). NOD2 protein is predominantly expressed in monocytes and monocyte-derived macrophages and dendritic cells, acts as a sensor for bacterial cell wall components and activates NF-κB that drives the inflammatory response. The CD-associated variants of *NOD2* reduce the ability of NOD2 to activate NF-κB, suggesting that loss of NOD2 function in the innate immune response results in increased bacterial survival and chronic inflammation (41). In addition, it has been shown that NOD2 stimulation induces autophagy in dendritic cells to prompt bacterial handling and generation of major histocompatibility complex (MHC) class II antigen-specific CD4+ T cell response, while this process is defective in CD patients. A similar effect is observed for *ATG16L1* and *IRGM* mutant cells (42). IL-23 is secreted by macrophages and dendritic cells. Interestingly, NOD2 was shown to have a positive regulatory effect on the IL-23/Th17 inflammatory pathway. Upon NOD2 stimulation, dendritic cells enhanced Toll-like receptor agonist-dependent induction of IL-23 and IL-1 production, which in turn promoted IL-17 expression in Th17 memory cells (43). Taken together, all four major genetic risk factors point to a compromised monocyte function in CD pathogenesis. Future studies focusing on these molecular pathways need to clarify the underlying mechanism(s) of diminished monocyte functions in CD pathogenesis and how 6-TG counteracts this primary immune defect.

Cross-ethnics IBD study

The recent GWA studies on Caucasian CD and UC have revealed dozens of genomic loci that are associated with IBD. Genes that are common for both diseases have been identified, as well as a significant number of unique susceptibility genes for either UC or CD. Combining the data from separate GWA studies now soon will lead to a saturation of the number of genes.
that significantly contribute to these diseases. The next step is to identify the genomic alterations (mutations/SNPs) that are responsible for disease development. Only for a few genes this is presently known. Interestingly, cross ethnic genetics may help to fine map the causal mutations, following a similar approach as we describe for the separation of the \( IL2 \) and \( IL21 \) locus as independent UC susceptibility genes. At the same time it is crucial that GWA studies are performed on patient cohorts of different ethnicities, especially in Asia. The cross ethnic genetic studies serve two main purposes. One is to determine whether the IBD patients from populations with high (e.g. Caucasian) and low (e.g. Asian) incidence share a similar profile of UC and/or CD susceptibility genes. Second, is to establish whether shared susceptibility genes between ethnicities contain the same causal variants, resulting in the same changes in proteins and/or biological pathways.

**Restore the immune balance of IBD patients**

The therapeutic effects of AZA in inflammatory bowel disease are its capability to enhance innate immunity and at the same time to induce apoptosis in intestinal T cells. This is consistent with the hypothesis of and impaired innate immunity and enhanced lamina propria CD4+ T cell resistance to apoptosis being the central pathogenic factors in these diseases (44, 45). This perception harbors important implications for the design of more specific therapeutic approaches, as AZA derivates with higher affinity to Rac1 may allow an even more efficient immuno-modulatory effect. Furthermore, there is evidence that a RhoA-dependent signaling pathway also plays a central role in inflammatory bowel disease, as RhoA and its corresponding effector are activated in Crohn’s disease and in experimental colitis. Specific blockade of Rho kinase significantly reduced intestinal inflammation in rats with TNBS-induced colitis, as inhibition of proinflammatory cytokine production via inhibition of NF-\( \kappa \)B activation was achieved (46). Therefore, blockade of Rho kinase may be a further therapeutic alternative in the treatment of inflammatory bowel disease. The specific inhibition of different Rho GTPases, like Rac1 and Rho, could have important implications in the treatment of IBD, as possible synergetic effects could enable an even more effective therapeutic approach.

To truly cure CD or UC, detailed knowledge is needed about the primary events, both genetic and environmental, that initiate the disease. GWA studies have given us important leads to follow up in experimental settings. However, for clinical treatment of CD and UC, e.g. inducing remission of intestinal inflammation, such detailed knowledge is not required, as in IBD the well-controlled balance of the intestinal immune system is disturbed at all levels to combat ongoing inflammation in existing lesions. The crucial thing in clinical treatment is to simply restore the immune balance, no matter at what level in the immune system this is achieved. Figure 1 shows a model of interaction in the human intestinal immune system. In my opinion, future research of IBD immunology should consider the interaction and regulation among the depicted components. Such an integrated approach is possibly more useful than the reductionist approach now favored by many investigators in the field. The complex interactions observed between immunology, genetics and medication as described in
In my opinion, this thesis certainly favors a holistic approach to fight CD and UC and, hence, I think future research should focus on employing systems biology-like approaches (including kinome profiling) as well as taking the individual genetic information about disease-associated alleles of the patient into account. Such studies may well provide the cornerstone for more effective treatment of IBD in the future.

*Figure 1.* According to Chinese Wu Xing philosophy (Taoism) which is the root of traditional Chinese medicine, the immune system of human intestine is a circle, where the interactions of five elements create a cycle (black, circle shaped arrows). When this cycle is disturbed during disease, novel interaction are induced to re-establish the cycle (white, star shaped arrows). Therapy can be directed at both the circular process or the disease-induced interactions.

**REFERENCE**


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