Chapter 4

Repertoire of IgA and IgG in inflamed and non inflamed ileum of Crohn's disease.

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Abstract

Among the most fundamental poorly understood issues in CD is the regional restriction of the disease. Areas not showing any obvious inflammation are lying next to areas with intense ulceration. To elucidate the molecular basis of this phenomenon we were interested to see whether we could obtain evidence for spatial differences with respect to the nature of the immune system. To this end we analysed the IgA repertoire in different biopsies samples from granulomatously inflamed tissues, and compared results to biopsy samples from normal tissue of different segments of the intestine (ileum) obtained from the same CD patients. We demonstrate marked differences in the immune repertoire between inflamed and inflamed parts of the intestine. Thus the spatial restriction of the disease is reflected in a spatially different identity of mucosal system. Evidently, such spatial restriction may be an important factor into explaining why Crohn's disease has a highly localised phenotype.

4.1 Introduction

Inflammatory bowel disease (IBD) is a chronic disease of the digestive tract, and usually refers to two related conditions, namely ulcerative colitis and Crohn's disease (CD). CD is characterized by a chronic-intermittent transmural, segmental, and typically granulomatous inflammation of different segments of the intestine mainly located in the small intestine (ileum) and or in the colon. Although the pathogenesis of IBD remains elusive, it appears that there is an exaggerated chronic activation of the immune and inflammatory cascade in genetically susceptible individuals. In this sense, it is well described that the mutation in the Nod2 gene of subgroup of CD patients patient leads to the activation of immune competent cells and uncontrolled chronic mucosal inflammation (Braat *et al*., 2005). In the last years, there is increasing experimental evidence that this deregulated immune reactivity against luminal bacteria plays a major role in the induction and perpetuation of this pathology (Podolsky, 2002).

Although insight into the pathogenesis of Crohn's disease increases rapidly (e.g. (Comalada and Peppelenbosch, 2006)), it is still not fully understood why the mucosal immune response is over-reactive in patients with this intestinal condition. However, it has become more and more evident that alterations in innate immunity are crucial in the pathogenesis of CD and involve a deregulated Th1 lymphocyte response (Canto *et al*., 2006;Romics, Jr. *et al*., 2006). Although $CD4^+$ T cell subset functions have been studied in great detail, the contributions of other immune cells to the development of IBD are just beginning to be understood. In particular, irregularities in B cell development and antigen-specific immunoglobulin production may be critical for understanding the pathogenesis of IBD. A abnormal immunoreactivity of serum or mucosal antibodies toward enteric bacterial flora has been reported in both animal models and IBD patients(Brandwein *et al*., 1997;Macpherson *et al*., 1996;Olson *et al*., 2004;van der Waaij *et al*., 2004). In CD patients, it has been reported a large relatively increase in the number of IgG-secreting cells (especially of the IgG1 subclass)(Dorn *et al*., 2002) associated with the lowest frequency of IgM memory B cells (Di Sabatino *et al*., 2004) and IgA^+ plasma cells. Moreover, a variety of autoantibodies, including ANCA, anti-erythrocyte antibodies, pancreatic antibodies, lymphocytotoxic antibodies, anti-Saccharomyces cerevisiae antibody (ASCA) and antibodies to epithelial cell components also have been described (Seibold *et al*., 1996). The importance of these autoantibodies in the pathogenesis of CD is unclear. It has been suggested that antibodies with specificity for epithelial cell components mediating antibody-dependent cellular cytotoxicity or antibodies involved in epithelial deposition of activated complement can contribute to mucosal injury in CD (Seibold *et al*., 1996).

The IgA isotype, which is considered as a mucosal immunoglobulin plays a critical role in preventing the intestinal invasion of both pathogenic and commensal bacteria (Macpherson *et al*., 2001). However, the chronicity of IBD lesions implies failure of antigen removal with constant activation of various non-specific biological amplification mechanisms. This unfavourable development involves sustained inflammation and tissue damage, featured as severely altered homeostasis and defective mucosal barrier function (Brandtzaeg, 2006). In active colonic IBD lesions there is also a shift from the IgA2 to the less stable IgA1 subclass (Kett and Brandtzaeg, 1987); more than 50% of the increased mucosal $IgA1^+$ plasma cells subset is J-chain deficient therefore producing monomeric IgA that cannot be translocated externally by pIgR (Brandtzaeg and Korsrud, 1984) and (Kett *et al*., 1988). The same happens with expanded IgA2-producing plasma cells with a substantial fraction (25–35%) (Kett *et al*., 1988). Thus, it seems evident that in IBD a less restricted leukocyte extravasation due to a changed endothelial adhesion pattern allows B cells expressing characteristics of systemic immunity to enter the lamina propria together with ordinary inflammatory leukocytes (Brandtzaeg, 2006).In this context, it is interesting to note that recent work of our group demonstrated that the pool of IgA production cells in the normal ileum are derived form a limited set of precursors and that only part of these cells shows signs of antigen selection, as suggested from a study of the IgA repertoire in healthy individuals (Yuvaraj *et al*, submitted).

Among the most fundamental poorly understood issues in CD is the regional restriction of the disease. Areas not showing any obvious inflammation are lying next to areas with intense ulceration. To elucidate the molecular basis of this phenomenon we were interested to see whether we could obtain evidence for spatial differences with respect to the nature of the immune system. To this end we analysed the IgA repertoire in different biopsies samples from granulomatously inflamed tissues, and compared results to biopsy samples from normal tissue of different segments of the intestine (ileum) obtained from the same CD patients. We demonstrate marked differences in the immune repertoire between inflamed and inflamed parts of the intestine. Thus the spatial restriction of the disease is reflected in a spatially different identity of mucosal system. Evidently, such spatial restriction may be an important factor into explaining why Crohn's disease has a highly localized phenotype.

4.2 Material and methods

4.2.1 Ileum biopsies.

After given informed consent (protocol approved by the medical ethical committee of the University Medical Center Groningen) terminal ileum biopsies were excised from the inflamed and non inflamed regions in 5 patients (Table-1). From patient 625 two biopsies were taken from periphery of the different ulcer and 2 biopsies from the center of the ulcer. PBS was added to all the biopsies and centrifuged at 1200 for 10 minutes. Later the supernant was used for gut microflora analysis and the tissues was digested trizol® for RNA isolation.

4.2.2 Total RNA extraction and RT-PCR

Total RNA was isolated from total ileum biopsies by the Trizol® method (Sigma Ltd Zwijndrecht, Netherlands) according to manufacturer's instructions. cDNA was produced by using Oligo-dT primers (Invitrogen, Breda, Netherlands) in a final volume of 30 µl and integrity of the cDNA was analyzed by β-actin PCR(Guikema *et al*., 2003). V_H primers used for different samples are as described by van Dongen *et al* (van Dongen *et al*., 2003). A 3' Cα common (IgA common) primer was designed that can be used for both IgA1 and IgA2 (GAATTCGAGTGGCTCCTGGGGGAA GA). On the basis of the sequenced PCR products both subtypes of IgA can be distinguished PCR was performed as described previously (S.yuvaraj submitted). PCR was performed for 35 cycles using a 60-second denaturizing step at 94^0C, 60-second annealing step at 60^0C and a 60-second extension step at 72^0C. The product was loaded onto agarose gel and the ethidium bromide stained band was recorded.

Number	Patient number	Biopsy condition
1	477-1	Not inflamed
	477-2	Inflamed
	477-3	Inflamed
	477-4	Inflamed
2	509-1	Not inflamed
	509-1	Inflamed
3	513-1	Not inflamed
	513-2	Inflamed
4	516-1	Not inflamed
5	625-B1	Inflamed (periphery)
	625-B2	Inflamed (periphery)
	625-C1	Inflamed (center)
	625-C2	Inflamed (center)

Table-1: Biopsy sample from 5 CD patients.

4.2.3 Cloning and Sequencing IgA and IgG transcripts

PCR products from all the biopsies were obtained by amplification of cDNA using the 5' FR1 primer set in combination with a 3' IgA common primer. PCR products were gel purified using a gel purification kit (Invitek,Germany), cloned into InsTAclone vector (Fermentas). 25 positive clones were sequenced using an automated sequencing device as described(Guikema *et al*., 1999).

4.2.4 Analysis of rearranged V genes

Nucleotide sequences were compared with the IMGT databases of germline sequences (Giudicelli *et al*., 2006) and V_H-D_H-J_H regions were analyzed using IMGT Quest (Giudicelli *et al*., 2004) and Joinsolver (Souto-Carneiro *et al*., 2004). The lengths of the CDR3 domains of translated IgA heavy-chain transcripts were calculated as described previously (Rock *et al*., 1994). Replacement over silent mutation (R/S) ratios for FR and CDR were determined (Dammers *et al*., 2000). Sequences having identical VDJ rearrangements were defined as clonally related.

4.3 Results

We have studied the diversity of intestinal IgA and IgG heavy-chain transcripts obtained from ileum biopsies specimen of CD patients. IgA and IgG encoding RNA was amplified from the inflamed and non inflamed biopsy by PCR using specific primers. High signals of IgA and IgG RNA were observed from all the disease biopsies. The material allowed us to analyze the V_H family usage and evaluation of the relative propensity of IgA or IgG subclass of immunoglobulin production.

4.3.1 Estimation of the number of B-cell precursors giving rise to IgA and IgG producing cells.

Using the data from sequenced V_H genes we estimated the number of different B cell precursors that gave rise to the analysed IgA V_H gene sequences (in one biopsy). This is based on the number of sets of clonally related sequences compared to the total number of obtained sequences from a certain biopsy and how well we cover the diversity of the Ig V_H gene repertoire. This coverage of different precursors in our sample can range from 0% when every newly obtained sequence is unique to 100% when every newly obtained sequence represents a member of a

previously described B cell clone. Using Good's formula we calculated a minimal coverage in the samples for each biopsy, excluding all 100% identical sequences, and a maximal coverage when also identical V_H sequences were considered to be derived from different B cells (Appendix Table-2). The minimal coverage ranged from 0% to 100% while the maximum was from 0% to 95%. The number of B-cell precursors that gave rise to the IgA producing cells in one biopsy was estimated using Choe and Lee formula (Table-3) (Chao and Lee, 1992). The minimal coverage estimate resulted in 110 B-cell precursors, while the maximal coverage estimate yielded 231 B-cells giving rise to the IgA producing cells in each biopsy. Patient 477 had only 1 and 3 precursors respectively from two biopsies, which apparently developed into the the entire complement of IgA producing cells in these biopsies. Apparently, the number of B cell precursors yielding the IgA producing compartment is very limited and gives rise to IgA and IgG plasma cells both in inflamed and non-inflamed tissues (Table-4). Inflammatory B cells do not derive from a larger precursor compartment as observed in non-inflamed biopsies. Strikingly, the IgA producing B cell compartment derived from a much larger base of precursors when compared the set of IgA producing B cells.

4.3.2 Clonally expanded ileum IgA and IgG B cells are commonly circulated.

From each biopsy, 25 IgA sequences were analyzed and in total 12 biopsies were used. Of the 300 sequences, 243 sequences were productively rearranged (Table-3). 41 sequences had 100% identical sequences resulting in 202 unique sequences. Some 100% identical sequences were observed from different biopsies within a single patient and could be assigned to different B cells, others were derived from the same biopsy not allowing assessment as to whether these identical sequences were derived from different B cells or whether we cloned multiple mRNA copies from a single B cell. In general, sequences are considered as clonally related if they have the same H-CDR3 region, i.e. the same VDJ joining. B cells with some mutations in the H-CDR3 regions can still be considered to be clonally related (Holtmeier *et al.*, 2000). Sequences with identical H-CDR3 regions are observed in biopsies taken from one person at inflamed and non-inflamed ileal sites, suggesting a common precursor for a fraction of the B cells seen in both the inflamed and non-inflamed tissue of the ileum. Fourteen sets of clonally related sequences that have most mutations in common, sequences shared between different biopsies, were obtained (see Figure-1). Thus global expansions of B cells in the mucosal tissue seems a common feature of antibody repertoire in CD patients.

4.3.3 More mutations are observed in non-inflamed tissue-derived ileal IgA genes as compared to inflamed tissue.

SHM mechanism increases the diversity of the immunoglobulin repertoire. In the IgA sequences obtained from CD patient biopsies, occurrence and nature of somatic mutations were analysed by evaluation of these sequences with the known human germline V_H sequences. Almost all IgA and IgG sequences display clear evidence for a high level of SHM, although two germline sequences were detected; one from inflamed region of diseased mucosa of patient 1 (sequence # 477-3 which represents an IgA) and the other was observed in the inflamed mucosa of patient 5 (sequence # 21.625-B1, representing a IgG isotype nucleotide sequence). Non inflamed biopsies show a significantly higher number of IgA gene mutations (average 20.5 mutations / 60 IgA sequences) when compared to non inflamed region-derived IgA genes (average 18.7/ 185 IgA sequences; $p<0.05$).

The ratio of replacement (R) to silent (S) mutations for CDR in this study were calculated as described elsewhere(Dammers *et al*., 2000). 38% of the sequences from non-inflamed biopsy showed a significantly higher ($p<0.05$) R/S ratio in the CDR regions whereas 54% of inflamed IgA sequences exhibited significant R/S ratio. This IgA response in the inflamed regions may be due to antigen selection pressure and may thus indicate in the tractus of CD patients local differences in immune system function exist.

4.3.4 Codon insertion and deletion is frequently observed in CD immunoglobulins.

In V_H gene, germline codons are deleted from the coding region and/or extra non-templated codons are inserted. In CD IgA and IgG sequences, insertions and deletions were often observed (Table-3) as compared to the non-inflamed tissue of the same patients ($P<0.05$), providing further evidence that in CD patients the tract area of disease is immunologically different from non-inflamed areas.

4.3.5 IgA1 is high in non inflamed tissue.

The newly designed 3' Cα primer successfully amplifies IgA1 and IgA2 subclasses and thus our experimental set up allows to discern between IgA1 and IgA2 usage in the inflamed and non-inflamed areas, respectively. In this study, IgA1 was vastly present in the non-inflamed tissue compared to the inflamed tissue. The utilization

of IgA1 between inflamed and non-inflamed biopsies was significantly different (p=0.01). On the other hand IgA2 was 20% more in inflamed tissues (Figure-2).

Sample	Productively rearranged sequences	Estimated number of classes	95 % CI
477-1	24	149.9	[33.4 , 681.7]
477-2	22	1.0	---
477-3	19	181.1	[23.7 , 1400.9]
477-4	18	3.1	[1.2 , 13.8]
509-1	14	---	
509-2	18	60.8	[14.9 , 255.4]
513-1	22	231.0	[33.2 , 1619.9]
513-2	21	---	
516-1	21	---	
625 B1 IgA	22	---	
625 B2 IgA	23	---	
625 C1 IgA	17	---	
625 C2 IgA	22	69.7	[26.8 , 186.2]
625 B1 IgG	24	60.0	[27.8 , 139.0]
625 B2 IgG	24	154.1	[37.2 , 647.1]
625 C1 IgG	21	---	
625 C2 IgG	24	56.9	[22.0 , 152.5]

4a

Sample	Productively rearranged sequences	Estimated number of classes	95 % CI
477-1	20	93.9	[23.6, 382.0]
477-2	1	---	
477-3	15	---	
477-4	2	---	
625 C2 IgA	21	99.8	[28.0 , 361.9]
625 B1 IgG	22	110.0	[30.7 , 401.4]
625 B2 IgG	20	---	
625 C2 IgG	18	---	

4b

Table-4: Estimation of B-cell precursors. 4a-Including 100% identical sequences 4b- Table excluding the 100% identical sequences

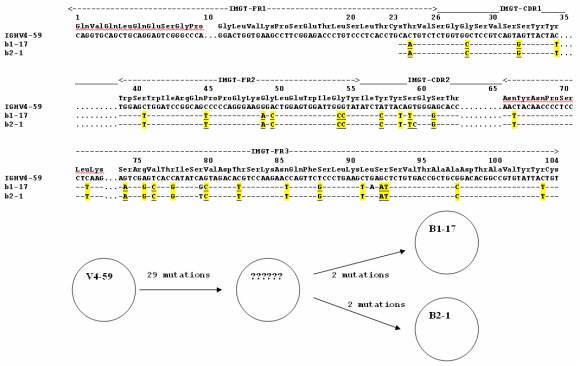

Figure-1: Alignment of clonally related IgA $V_HD_HJ_H$ sequence derived V_H genes from ileum and genealogical trees deduced from the mutations observed in the V_H genes of these clones. Sequences of the germline gene are shown on top. Dashes indicate identical nucleotides, and gaps resulting from IMGT subdivision are marked by dots. Replacement mutations are shown in bold/underscored characters.

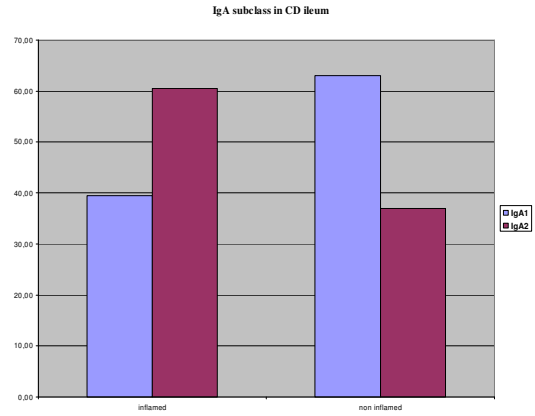

Fig. 2 IgA1 and IgA2 subclasses usage among the IgA sequences.

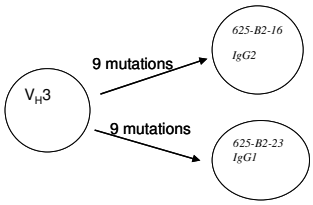

Fig. 3 Evidence IgG sequential switching from IgG1 to IgG2

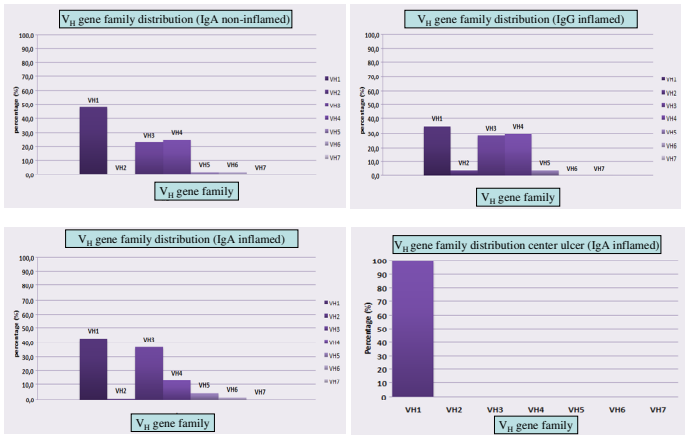

Fig. 4 V_H usage among the IgA and IgG sequence of inflamed and non-inflamed ileum biopsies of Crohn's diseased patients.

4.3.6 VDJ usage is reduced in the inflamed tissue.

V_H1 is highly used in the mucosal tissues especially in ileum(S. Yuvaraj submitted). Similarly, in the non-inflamed tissue, IgA sequences utilize around 48% V_H1 (see Figure-4) family members, followed by V_H4 and V_H3. In the case of inflamed tissue, V_H3 is relatively more prevalent as compared to non-inflamed tissue, but also in this tissue V_H1 usage predominates. All the IgA sequences analyzed from patient 4, biopsy # 625-C1 and biopsy # 625-C2 (both biopsies taken from the center of the ulcer) use V_H1 genes. In the IgG sequences, the use of V_H1, V_H3 and V_H4 genes is roughly equal, but V_H1 usage is more often observed. Not much difference is observed in the D_H gene usage when inflamed and non-inflamed tissue is compared. In a similar manner we observed the same amount of usage of D_H3 genes in the normal ileum. J_H usage remains the same through out the different tissues, with J_H4 being the most commonly used member in the intestine of CD patients.

4.3.7 Evidence IgG sequential switching from IgG1 to IgG2.

In general, during class switching recombination (CSR) IgM^+ B-cells changes their constant region to other isotypes such as IgG, IgA or IgE (Stavnezer, 1996). In the present study, for the first time we observe in inflamed ileum tissue that IgG1 and IgG2 constant regions can be derived from the same precursor (see Figure-3). In one set of 100% identical sequences, one sequence uses the IgG1 constant region while the other sequence uses the IgG2 region. The underlying mechanism for this sequential switching of IgG region use is not known.

4.4 Discussion:

CD shows a discontinuous, transmural inflammation pattern and can appear in the whole alimentary tract, and preferably affects the ileum. The peculiarity in CD is the presence of spatially restricted inflamed and non-inflamed regions in the diseased organ; the reason for this pattern remains unclear but presents one of the most fundamental questions in contemporary CD research. In an effort to obtain more insight into this issue, we analyse in the present study the IgA and IgG repertoire from biopsies taken from inflamed and non-inflamed terminal ileum. 60% of the IgA and IgG sequences were selected in the inflamed region when compared to the non-inflamed region. Although statistically not significant, there was a clear trend for more mutations in IgA and IgG sequences in non-inflamed tissue when compared to inflamed regions. The most straightforward explanation is

that CD is an ongoing immune response with more antigen selection during the biogenesis of IgA and IgG plasma cells.

Immunoglobulin repertoire of the inflamed region was further investigated to get more insight whether local differences in the immune response could play a role in explaining the hallmark spatial restriction of CD. To end we analysed IgA and IgG sequences from both the rim and from the central part of the inflamed region. Importantly, clonally related sequences also shared between these two regions and also between different ulcers. 8 sets of the 100% identical sequences and 2 sets of clonally related sequences were observed in IgG sequences which was relatively low in the IgA sequences in patient-4. Thus B cells involved in the disease undergo substantial expansion before they enter the mucosa. There is clear bias for these clonally-related sequences to end up in inflamed tissue, our data provide strong evidence that immunological functioning is fundamentally different in inflamed versus non-inflamed regions of the gut in CD. Whether these differences actually also explain the spatial restriction of the disease or whether these differences are a consequence of the disease remains subject, however, for further experimentation.

An estimation of the number of precursors can be made from the ratio of unique verses non-unique sequences. First, the coverage can be calculated as the percentage of clonally related sequences among the total number of sequences within a single biopsy(Good, 1953). Coverage percentages ranging from 0% to 99% were obtained. From these coverage's the actual number of B cell precursors that gave rise to the IgA producing cells in each biopsy was estimated. For these calculations we did not include the sets of clonally related cells that were shared between different biopsies, but we considered each biopsy as an individual experiment. A surprisingly high number of about 190 B cell precursors gave rise to all IgA producing cells in non-inflamed biopsy and low number of around 73 precursors from inflamed tissues. This indicates that there are few precursors that give rise to the entire IgA produced within the inflamed tissues. This is best illustrated by the large number of identical 24/24 and 18/24 sequences we obtained from two inflamed biopsies. Such areas must be dominated by largely expanded B cell population. We did not observe shared CDR3 regions between IgG and IgA sequences. This indicates that the IgA and IgG plasma cells were not derived from the same precursors.

In general SHM was observed in almost all sequences of IgA as well as in IgG obtained from the human mucosa like we observed previously in that healthy volunteers. (Yuvaraj *et al* submitted) In addition to base-pair substitutions, there are several reports of antibodies in which germline V-gene codons have been deleted/inserted from the coding region. Such insertions and deletions have been

shown to occur in several human B cell malignancies, in GC B cells, in human hybridomas, and in peripheral blood. Reason *et al*. (Reason and Zhou, 2006) have recently shown that insertion/ deletion of codons occurs as a normal part of the somatic maturation of the human antibody response.

The presence of microflora in the inflamed region is higher and there is a difference in the bacterial composition between inflamed and non-inflamed biopsies (Bibiloni *et al*., 2006). Importantly, a preliminary investigation into the bacterial composition of the same biopsies as we used for characterising immunoglobulin repertoire in the present study demonstrated profound differences in flora composition between inflamed and non-inflamed samples (M. Comalada and S. Yuvaraj, unpublished observations), the diseased areas also containing more bacteria. This observation correlates well with our results on IgA repertoire differences between inflamed and non-inflamed biopsies which involve clonal expansion and selection of IgA producing cells in the inflamed region. It was reported that microflora play an important role in the development of mucosal immune system, especially to increase the IgA producing cells (Bos *et al*., 2005) and the present study seems to reflect this observation. Again, however, it is difficult to distinguish whether locally the flora is different because of altered local immunity or whether altered local immunity facilitates the establishment of an alternative flora.

In normal ileum, less than half of the IgA plasma cells show signs of antigen selection as eidenced by significant R/S ratio's (Yuvaraj *et al*, submitted), while the remaining IgA plasma cells do not show signs of antigen selection in spite of a high number of somatic mutations. Non-inflamed tissues of CD patients represent a IgA repertoire that has the same characteristics as that observed in the mucosa of healthy volunteers. In contrast, affected areas in CD patients show a higher number of sequences displaying evident selection, that as discussed above could probably be directed towards the locally different microflora constituents Nevertheless, both normal as well as inflamed mucosa has strong prevalence of V_H1 usage, further supporting our previous finding that this V_H gene family is preferentially used in mucosal tissue. Surprisingly, within ulcerated areas spatial differences in the development of immunorepertoire are observed: all the IgA sequences from the center of the inflammation have utilized V_H1 family, whereas this was not observed at the rim of the ulceration. We interpret this finding as further evidence that important regional differences in the intestine of CD patients with respect to immunoregulation exist.

In conclusion, in the present study for the first time we present evidence that the immune system is developing differently at the inflamed lesions in CD as compared to uninflamed tissue of the same patient (which seems indistinguishable from that of healthy volunteers in this respect). Further research should now

provide insight as to whether this regionality in immunoregulation is causative for CD and actually explains the spatial restriction of the disease or whether this is a reflection of deeper pathological mechanism at ulcerative sites.

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