UNIVERSITAT DE BARCELONA

Immune mechanisms involved in inducing remission in Crohn's disease patients undergoing hematopoietic stem cell transplant

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"Immune mechanisms involved in inducing remission in Crohn's disease patients undergoing hematopoietic stem cell transplant"

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A mis abuelos,
siempre en mi corazón.

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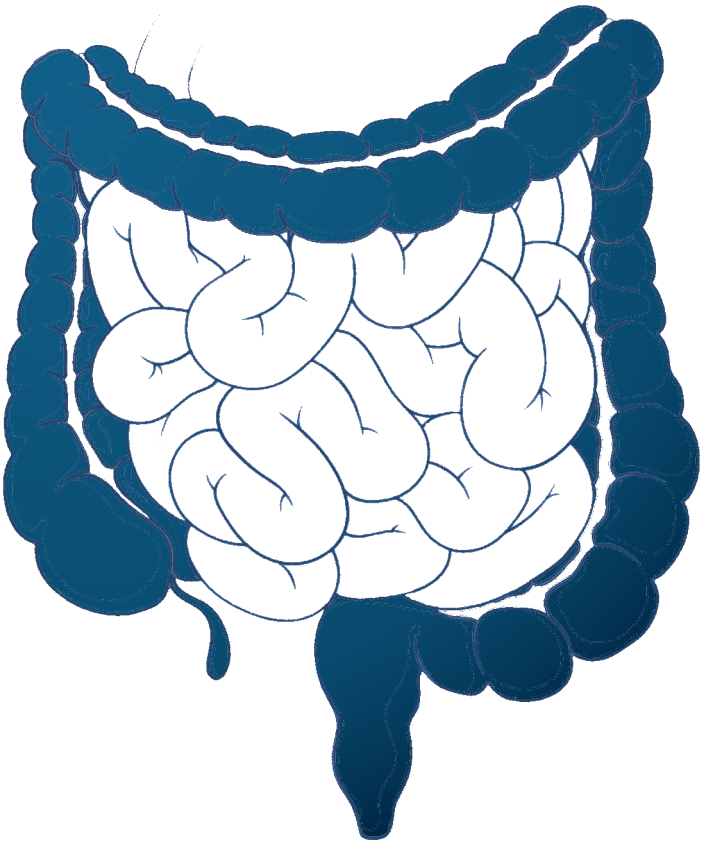
Abbreviations

ADWP	autoimmune disease working party
AIEC	adherent-invasive pathogen *Escherichia coli*
Anti-TNF	anti-tumor necrosis factor α
AMCA	anti-mannobioside carbohydrate antibodies
AMP	antimicrobial protein
ANCA	anti-neutrophil cytoplasmic antibody
APCs	antigen-presenting cells
ASCA	anti-*Saccharomyces cerevisiae* antibodies
ATG	anti-thymocyte globulin
BCR	B cell receptor
Breg	regulatory B cell
CDAI	Crohn's disease activity index
CDEIS	Crohn's disease endoscopic index of severity
CDR	complementarity-determining region
CI	confidence interval
CNT	controls
Cy	cyclophosphamide
DC	dendritic cell
DN	double negative T cell
DNA	deoxyribonucleic acid
DP	double positive T cell
EBMT	European Group for Blood and Marrow Transplantation
EMA	European medicines agency

FACS	fluorescence-activated cell sorting
FC	fold change
FDA	food and drug administration
FDR	false discovery rate
FGF-2	fibroblast growth factor 2
G-CSF	granulocyte colony stimulating factor
GRO	growth-regulated oncogene
GWAS	genome wide association analysis
HIV	human immunodeficiency virus
HSCs	hematopoietic stem cells
HSCT	hematopoietic stem cell transplant
IBD	inflammatory bowel disease
IBDQ	inflammatory bowel disease questionnaire
IFN	interferon
Ig	immunoglobulin
IGH	immunoglobulin heavy chain gene
IL	interleukin
ILCs	innate lymphoid cells
IMD	immune-mediated diseases
IPA	ingenuity pathway analysis
MCP	monocyte-chemotactic protein
M-H	Morisita-Horn index
MHC	major histocompatibility complex
MMPs	matrix metalloproteinases
MSCs	mesenchymal stem cells

NHEJ	non-homologous end joining
NK	natural killer cells
NSAIDs	non-steroidal anti-inflammatory drugs
OD	optical density
OR	odds ratio
PAMPs	pathogen-associated molecular patterns
PCA	principal component analysis
PD-1	programmed cell death-1 protein
PRR	pattern recognition receptors
RAG	recombination-activating gene
RNA	ribonucleic acid
RNAseq	ribonucleic acid sequencing
RTE	recent thymic emigrants
SEM	standard error of the mean
SES-CD	simplified endoscopic severity index for Crohn's disease
SP	single positive T cell
TCR	T cell receptor
TGF	transforming growth factor
Th	T-helper cell
TLRs	toll-like receptors
TNF	tumor necrosis factor
TRECs	T cell receptor excision circles
TReg	regulatory T cell
TRM	resident memory T cell
TT	Tetanus toxoid

Introduction



Chapter 1: Crohn's disease

Crohn's disease is a chronic inflammatory disorder characterized by alternating periods of remission and clinical relapse that mainly affects the gastrointestinal tract, although it is frequently associated with extraintestinal manifestations and/or concomitant immuno-mediated diseases. It is characterized by a transmural and discontinous inflammation and while it can affect the entire gastrointestinal tract from the mouth to the anus, the ileum and colon are the most commonly affected areas (Figure 1).[1]

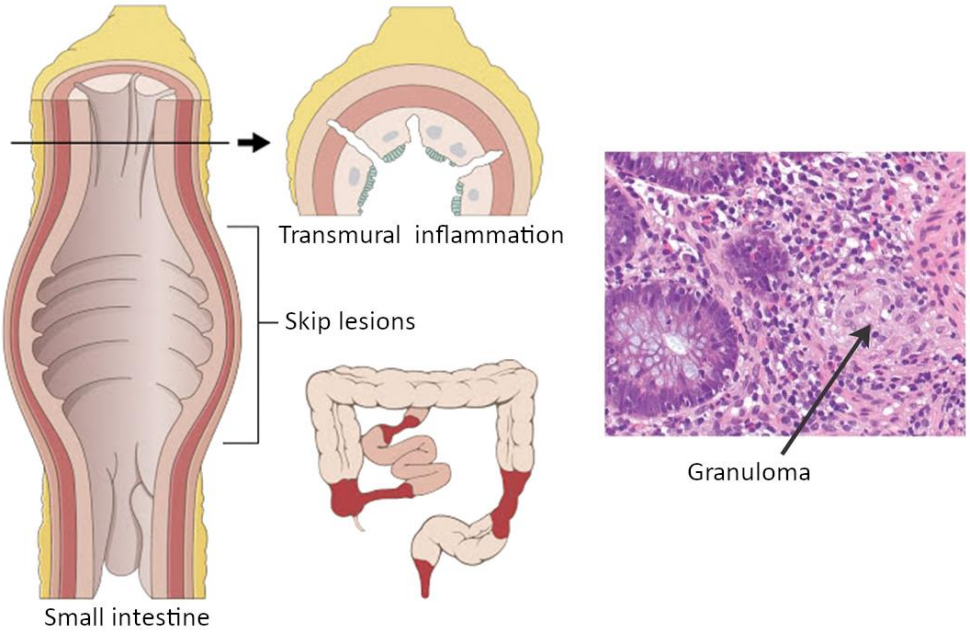


Figure 1. Schematic representation Crohn's disease main characteristics. Adapted from Kumar *et al.*[1]

Clinical presentation

Crohn's disease is a heterogeneus entity and can involve diarrhea, abdominal pain, weight loss, as well as systemic signs, with malaise, anorexia and fever being the most common symptoms.[2] Extraintestinal manifestations such as arthritis, skin disorders, primary sclerosing cholangitis, venous or arterial thromboembolism and/or pulmonary involvement are also associated with the disease.[3]

The clinical heterogenicity of Crohn's disease has lead to the development of the Montreal classification[4] system whereby patients are divided according to their

age of disease onset, anatomical disease location and disease behaviour (Figure 2). The location of Crohn's disease according to Montreal classification (ileal, colonic or ileocolonic) remains stable over time after diagnosis. In contrast, behavioral phenotypes change over time, with most patients progressing from an inflammatory phenotype to a stricturing or penetrating one.[3, 5]

Disease course

Crohn's disease usually follows a relapsing and remitting course, particularly in early disease. Relapses are characterized by the clinical symptoms previously described. According to population-based data from 1935-2008, only 10% of patients

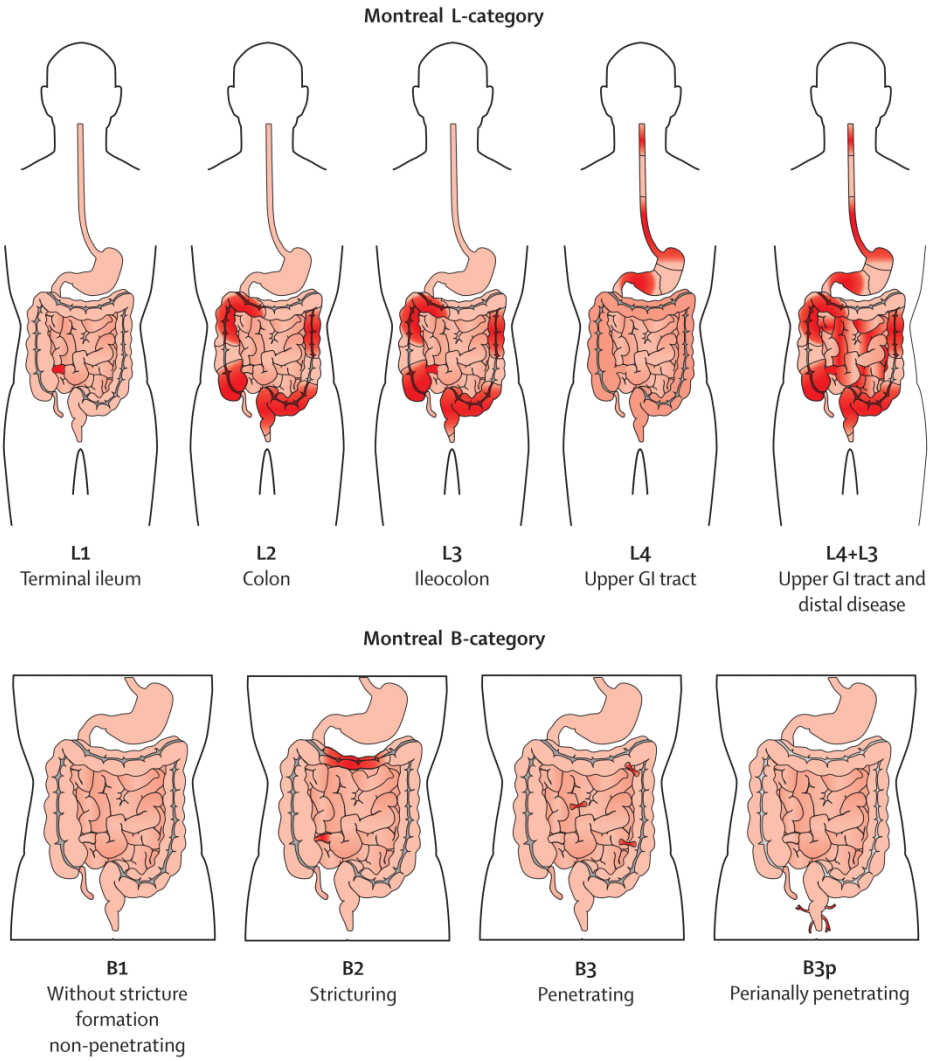


Figure 2. Montreal Classification. Adapted from Baumgart *et al.* [3]

have prolonged clinical remission.[6] Even during periods of clinical remission, the bowel may not be free of lesions; subclinical inflammation often persists. The disease can evolve to fibrostenotic stricture or penetrating lesions (fistula and abscess) of the bowel, resulting in structural bowel damage. According to this data, 50% of adult patients with Crohn's disease have an intestinal complication within 20 years after diagnosis. Surgical bowel resection is required in 50% of Crohn's disease patients to treat such lesions. Following surgery, the risk of post-operative recurrence is 44-55% after 10 years, leading to progressive loss of intestinal function and disability.

Epidemiology

There are large variations in the incidence of Crohn's disease among the world's various geographic regions. Its incidence has been traditionally higher in North America (up to 20.2 per 100,000) and in Western Europe (up to 12.7 per 100,000). However, the incidence of Crohn's disease is currently increasing in emerging populations such as in Asia. Within a defined geographical area, incidence rates and patterns of change are heterogeneous. For instance, incidence is higher in urban than in rural populations. Such epidemiologic observations indicate that there are strong environmental influences on Crohn's disease. It is also known that while it can affect people of all ages, the most common age onset is between the second to fourth decade of life and some studies suggest a modest second peak between the sixth and seventh decades of life.[7]

Diagnosis of Crohn's disease

A single gold standard test for the diagnosis of Crohn's disease is not available.[2] Crohn's disease is diagnosed by a combination of clinical, laboratory, endoscopic, histological and imaging findings. There are no specific signs at physical examination suggestive of Crohn's disease. Laboratory studies can reveal diverse biomarkers that will be described in the following section. A stool specimen can be taken for culture; the detection of parasites or bateria, such as *Clostridium difficile*, can rule out an infectious disease. The detection of fecal calprotectin, a neutrophil

cytosolic protein, is a good marker of endoscopic activity, with sensitivity around 70-80% and specificity of 80-97%, depending on the cut-off concentration used.[8] Endoscopic findings can include colonic or ileal inflammation or ulceration and rectal sparing. These lesions are typically discontinuous. Histological findings can confirm a diagnosis based on the presence of acute or chronic inflammation. For example, granulomas, which historically have been considered characteristic histological lesions of Crohn's disease (Figure 1), are found in up to 30% of surgical specimens.

Assessment of activity in Crohn's disease

Crohn's disease is associated with reduced quality of life and fertility, as well as increased morbidity, hospitalization and surgery rate. As mentioned above, its clinical presentation is heterogeneous. The achieving of clinical remission does not correlate to the absence of mucosal inflammation, nor does it determine the clinical course of the disease or prevent long-term consequences.[9] Therefore, treatment decisions should be based on more objective measures of disease activity. In clinical studies, assessments of Crohn's disease are currently based on the following aspects:

Clinical activity

The most widely used index of activity in clinical trials has been the validated Crohn's disease activity index (CDAI). It collects clinical data from a 7-day patient diary, including patient-reported stool patterns, average abdominal pain, general well-being, complications such as fistulas, abscesses or fevers, findings of an abdominal mass, hematocrit values and weight. It divides the clinical severity of the disease into the following categories: **quiescent** (CDAI <150), **mild** (CDAI 150-220), **moderate** (CDAI 220-450) and **severe** (CDAI >450).[2]

Endoscopic activity

Endoscopic activity can be measured by the Crohn's Disease Endoscopic Index of Severity (CDEIS) in cases of ileal and/or colonic activity. The CDEIS collects data on the presence of superficial and deep ulcers, stenosis, as well as those mucosal surface affected by disease and ulceration in all colonic segments.[10] Daperno and colleagues presented the Simplified Endoscopic Severity Index for Crohn's Disease (SES-CD, Table 1).[11] This index evaluates the penetration and size of ulcerations, which are known to reflect the severity of Crohn's disease. The SES-CD has excellent inter-observer agreement for all selected variables (kappa coefficient 0.79-1.00). It is also correlated to a considerable degree with the CDEIS (r = 0.92).[11]

Variable	Simple Endoscopic Score for Crohn's Disease values			
	0	1	2	3
Size of ulcers	None	Aphtous ulcers	Large ulcers	Very large ulcers
Ulcerated surface	None	<10% (ø 0.1 to 0.5 cm)	10-30% (ø 0.5 to 2 cm)	>30% (ø > 2 cm)
Affected surface	Unaffected segment	<50 %	50-75%	>75%
Presence of narrowings	None	Single, can be passed	Multiple, can be passed	Cannot be passed

Table 1. Definitions of simple endoscopic score for Crohn's disease from Daperno *et al.*[11]

Biomarkers

Laboratory investigations should examine serum C-reactive protein, which is useful for monitoring disease activity (although it lacks specificity for intestinal inflammation[12]) and for guiding therapy and short-term follow-up. Measurements of the erythrocyte sedimentation rate should be used for those patients in whom serum C-reactive protein does not significantly increase in response to inflammation. One of the most recurrent complications of Crohn's Disease is anemia, which may affect patients' quality of life and therefore should be evaluated at initial diagnosis. Besides hemoglobin and leukocytosis, signs of iron, folate and B12 vitamine deficiency are evaluated through a full blood count.

Imaging techniques

The role of imaging in patients with Crohn's disease arose from the need to evaluate portions of the small bowel inaccessible to optical endoscopy. Magnetic resonance enterography is the preferred imaging modality for those patients with small bowel involvement or complications, since it can accurately detect intestinal involvement in Crohn's disease, including extramural complications, without radiation exposure.[13]

Quality of life

Health-related quality of life can be evaluated by the Inflammatory Bowel Disease Questionnaire (IBDQ). This consists of 32 questions grouped into four categories: bowel, systemic, social and emotional.[14] According to Alrubaiy and colleagues,[15] IBDQ has the strongest published evidence of reliability and validity and is well established in the literature for assessing quality of life in inflammatory bowel disease (IBD) patients.

Etiology and pathogenesis

While the etiology of Crohn's disease remains an enigma, several environmental, genetic and immunological factors may contribute to its onset and progression. Some have suggested that Crohn's disease results from an aberrant over-reactive mucosal immune response, leading to loss of the homeostasis between bacterial antigens and the immune system. A breakdown in the mucosal barrier could promote antigen translocation into the lamina propria and an aberrant inflammatory response against harmless components of the intestinal flora (Figure 3).[16, 17, 18] Therefore, the disease must be regarded as multifactorial in nature, and many aspects should be taken into account.

Environmental factors

Several environmental factors have been studied in the pathogenesis of Crohn's disease. Smoking is perhaps the most exhaustively documented. A meta-analysis quantified the increase in risk associated with smoking to be twofold for Crohn's disease (OR 1.76, 95% CI 1.40–2.22).[19] Active and passive exposure to smoking in infancy has comparable effects.[20] Furthermore, patients who smoke have a higher risk of developing severe disease.[7] The pathogenic role of smoking is possibly explained by the influence it has on the microbiota,[21] its modification of endothelial function[22] and alterations to smooth muscle tone[23] exerted in the gut.

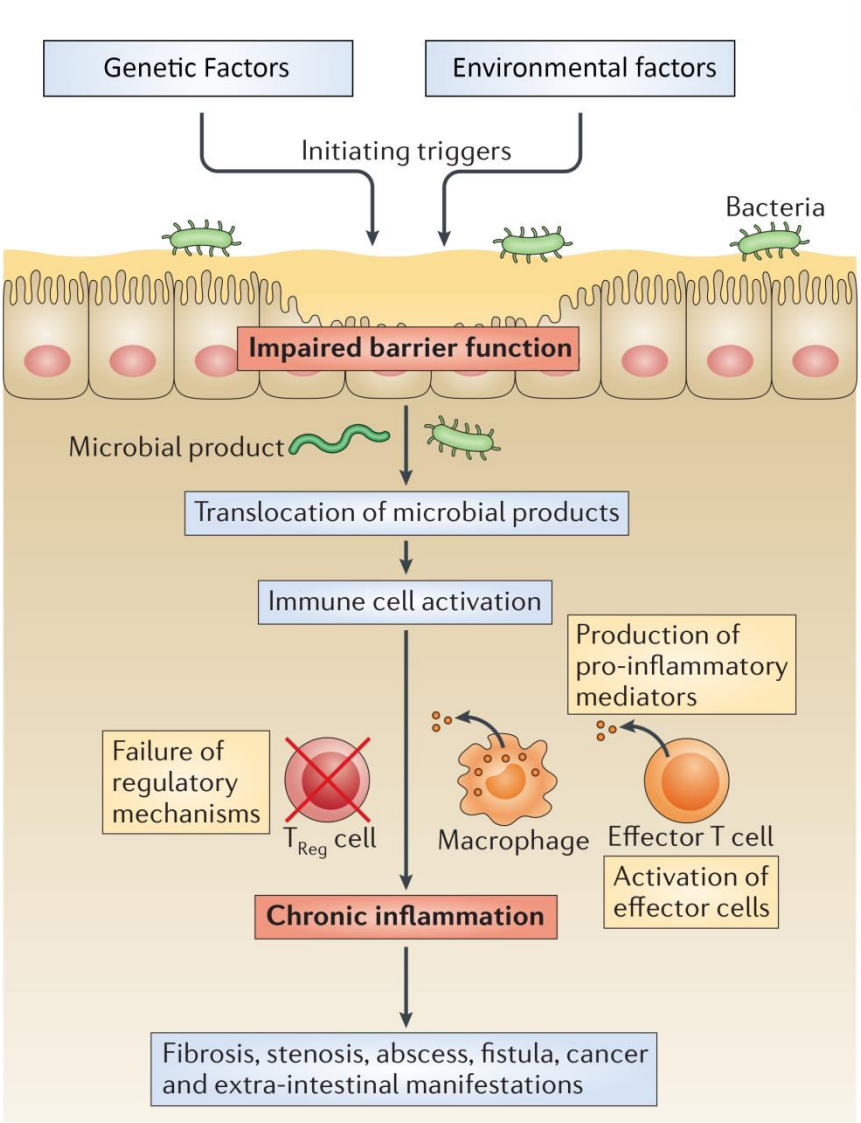


Figure 3. Conceptual scheme for the pathogenesis of Crohn's disease. Adapted from Neurath *et al.* [18]

Stress, infection and the use of non-steroidal anti-inflammatory drugs (NSAIDs) or antibiotics are additional factors that might play important roles in the pathogenesis of Crohn's disease. Stress modifies secretion and visceral sensitivity and motility, resulting in the production of pro-inflammatory cytokines, activation of macrophages and alterations to intestinal permeability and microbiota.[24] Infections and NSAIDs can induce temporal unspecific inflammation and play a role in the pathogenesis of Crohn's disease.[25, 26] Antibiotics induce a perturbation of the microbiota, and if used during early childhood can increase susceptibility to Crohn's disease, as has been observed in Western populations.[7] Unfortunately, the exact mechanisms by which these factors trigger or reactivate quiescent Crohn's disease are not fully understood.

Genetic factors

The inheritance pattern of Crohn's disease is unclear. Nevertheless, this condition tends to cluster in families, and having an affected family member is a significant risk factor for the disease.[7] A large number of Crohn's disease susceptibility loci have been identified through the combined application of linkage analysis, fine mapping studies, candidate-gene studies, and genome-wide association analyses (GWAS).[27, 28] Genetic association analysis has implicated several key pathways that are crucial for intestinal homeostasis, including barrier function (i.e., *IBD5*, *DLG5* and *XBP1*), pattern recognition receptors (i.e., *NOD2*, *TRL4*, *CARD9*) and the differentiation of Th17 cells (i.e., *IL23R*, *JAK2* and *STAT2*) among others.[28, 29]

Microbiota

In the gut a massive and diverse community of microbes circulates in proximity to the epithelial cell surface. The unhealthy balance of bacteria, as well as the abnormal communication between gut microbial communities and the host immune system at the mucosal barrier, has been identified as the core defects leading to chronic intestinal inflammation.

Dysbiosis, which is the global alteration of the gut microbial community, has been reported in Crohn's disease during the last 10 years.[30, 31] Crohn's disease is associated with lower microbial diversity and certain species may play specialized roles in the disease. One promising potential causative agent in Crohn's disease is the adherent-invasive pathogen *Escherichia coli* (AIEC). Darfeuille-Michaud and colleagues found AIEC strains in 21.7% of ileal Crohn's disease chronic lesions compared with only 6.2% of healthy controls.[32]

By contrast, some microbial species may act as protective commensals. As just one example, a decrease in *Faecalibacterium prausnitzii*, a butyrate-producing bacteria belonging to the *Firmicutes* phylum, is associated with Crohn's disease[31, 33] and its abundance inversely correlates with endoscopic recurrence at 6 months.[34]

Intestinal immunologic response

Crohn's disease presents altered innate and adaptive immune responses; particularly a loss of tolerance to commensal bacteria has been suggested as the underlying mechanism.[35, 36, 37] The diverse pathways that are altered as a result will be discussed in next chapter.

In short, a disruption of the innate defense occurs, leading to antigen translocation into the lamina propria. Subsequently, an aberrant immune response develops against harmless components of the intestinal microbiota. Crohn's disease pathogenesis is clearly associated with exacerbated adaptive immune responses in the gut. This imbalance in the T cell response leads to an increase in the production of pro-inflammatory cytokines and mediators.

Chapter 2: immune response in Crohn's disease

The intestinal mucosa contains the largest reservoir of immune cells in the body[38] and constitutes the setting in which pathogenic events leading to Crohn's disease manifestations occur. We can separate the response of the immune system by its two branches: innate and adaptive.

Innate immunity

Innate immunity comprises defense mechanisms mediated by the evolutionarily more primitive components of our immune system. These include physical barriers, such as the skin, eyelashes and the epithelial barrier; myeloid cells, such as macrophages, dendritic cells, and neutrophils; and serum proteins such as those belonging to the complement and coagulation systems. The innate immune system can activate the adaptive immune response, principally via antigen-presenting cells (APCs). Innate responses are, therefore, the first line of defense encountered by invading infectious agents and are characterized as being rapid, non-antigen-specific and by not conferring long-lasting memory. In homeostasis, the innate immune system in the gut is tolerant to dietary and microbiota antigens that are present in the intestinal lumen and in contact with the mucosa. Intestinal barrier disturbances and defective microbial sensing are thought to play a crucial role in the pathogenesis of Crohn's disease.

Epithelial barrier

The epithelial barrier is the largest of the body's mucosal surfaces, covering around 400m^2 of surface area with a single layer of cells organized into crypts and villi. A large proportion of the epithelial cells are absorptive enterocytes, which are in charge of metabolic and digestive functions. Nevertheless, the intestinal epithelium carries out a diverse array of functions besides digestion, which are performed by specialized cell lineages (Figure 4).[39] For example, goblet cells secrete a large, net-like polymer known as mucus.

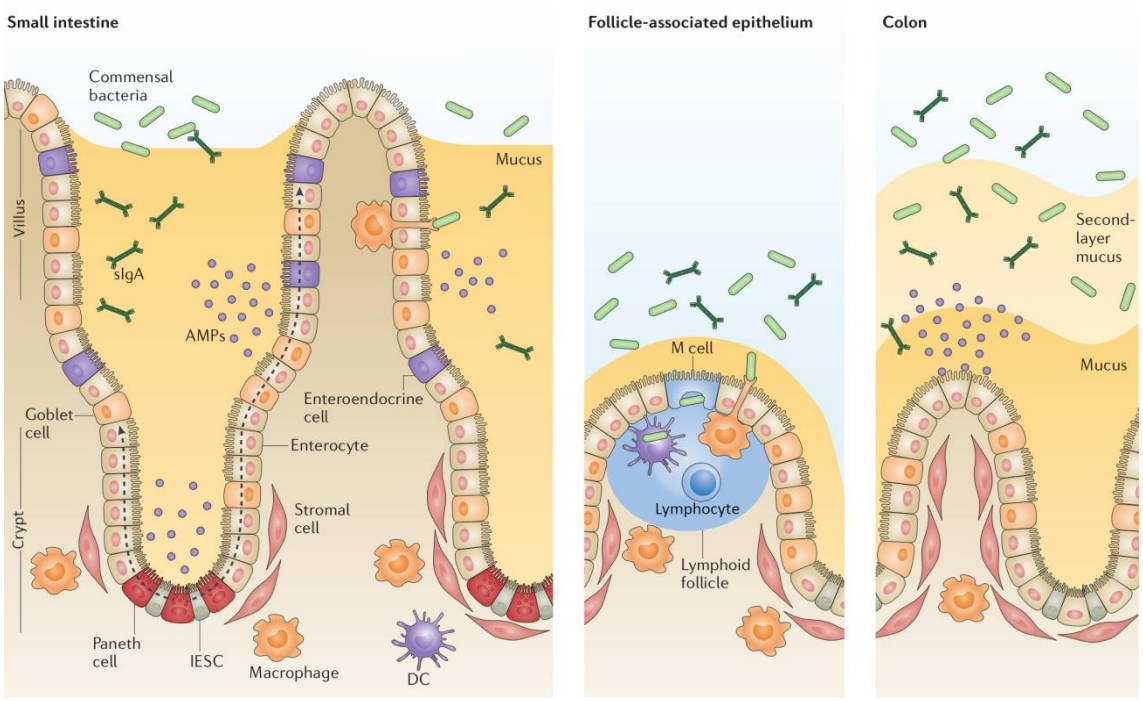


Figure 4. The intestinal epithelial barrier. Representation of the layer of mucus and cells and their distribution in the small intestine (left), follicle associated epithelium (middle) and colon (right). The intestinal epithelial stem cell (IESC) niche, containing epithelial, stromal and immune cells, controls the renewal of the epithelial layer. Differentiated intestinal epithelial cells migrate up the crypt–villus axis, as indicated by the dashed arrows, except for Paneth cells that stay at the bottom of the crypt. Paneth and goblet cells secrete antimicrobial proteins (AMPs) and mucus. Microfold (M) cells and goblet cells mediate transport of luminal antigens and bacteria across the epithelial barrier to dendritic cells (DCs), and intestine-resident macrophages sample the lumen through transepithelial dendrites. Adapted from Peterson *et al.*[39]

The first physical barrier that intestinal bacteria and food antigens encounter is the mucus layer covering the intestinal epithelium. Mucus slows down bacterial infiltration and is continuously renewed by the goblet cells, a process that pushes bacteria out towards the lumen. Furthermore, the mucus concentrates anti-microbial proteins (AMPs) secreted by Paneth cells and other secretive cells, generating a gradient from the epithelial cells out towards the lumen.[39]

The intestinal epithelium is in contact with the mucus layer and forms the second line of defense against food antigens and bacterial infections. Epithelial cells maintain the integrity of the epithelial barrier by intercellular junctions, such as adherens and tight junctions. The transytosis and luminal release of secretory IgA by epithelial cells reinforces the barrier function. Microfold cells and goblet cells allow for transport of microbes and particles across the epithelial layer to the lamina

Pathogen recognition

The accurate sensing of infectious agents is critical for the initiation of the innate response against pathogens. Immune cells of the innate system – not only macrophages or dendritic cells, but also non-immune cells, such as epithelial cells and myofibroblasts - recognize and respond to pathogens in a similar way. This recognition is mediated by pattern recognition receptors (PRR) that sense conserved structural motifs on microorganisms, known as pathogen-associated molecular patterns (PAMPs). NOD-like receptors are intracytoplasmic PRRs. NOD2 was the first risk gene to be identified as conferring an increased susceptibility to Crohn's disease.[40] The Nod2 receptor recognizes a peptidoglycan motif present in bacteria and drives the activation of the NF-κB signaling pathway. Functional studies revealed that Crohn's disease-associated Nod2 variants exhibit reduced or complete loss of activity compared to the wild-type protein.[41] Because activation of NF-κB plays a central role in the host response to infection by microbial pathogens, NOD2-mediated susceptibility to disease may stem from a failure to trigger the NF-κB pathway response.

Innate Immune Cells

Phagocytes (neutrophils, macrophages, monocytes and dendritic cells) are able to initiate a response against harmful agents detected by a specific class of PRRs known as Toll-like receptors (TLRs). Phagocytic cells secrete cytokines and chemokines and maintain homeostasis by removing dead cells, infected cells and microorganisms. Macrophages and dendritic cells are also APCs that interact and activate the adaptive immune system.

Other remarkable innate immune cells are innate lymphoid cells (ILCs), which are lymphoid effector cells without antigen-specific receptors and lacking markers associated with 'classic' immune cell lineages. There are three different groups of ILCs based on their phenotype and cytokine production profile. Natural killer (NK) cells are catalogued as ILCs1, and are found throughout the intestine, mostly expressing high levels of CD56 in healthy states In patients with Crohn's disease, ILCs (NKs and ILCs3) are enriched in the colonic lamina propria and cytotoxic NK cells express low levels of CD56. [42, 43]

Adaptive immunity

Adaptive immunity comprises those defense mechanisms mediated by T and B lymphocytes, and the specialized molecules required for their function. As opposed to the innate immune response, the adaptive immune system is highly specific and confers long-lasting immunity. It is also adaptable, since specificity for the antigen is the result of complex maturation and development of immune cells. Under normal circumstances, the components of the adaptive immune system cooperate with each other and with the innate immune system to mount an efficient response capable of eliminating the invading pathogens.

However, a deregulated response may lead to the onset of inflammation by an excessive release of chemokines and cytokines, which have multiple pathogenic effects on both branches of the immune system.

B cells

B cells perform several immunological functions (Figure 5). While their main function is antibody production, B cells also function as APCs and secrete cytokines. B cells have been shown to suppress the proliferation of effector $CD4^+$ T cells through the interaction of CD40 on B cells and its ligand (CD40L) on effector T cells.[44, 45] In addition, B cells may enhance the expansion of regulatory T cells either by antigen presentation or by the production of IL-10.[45, 46]

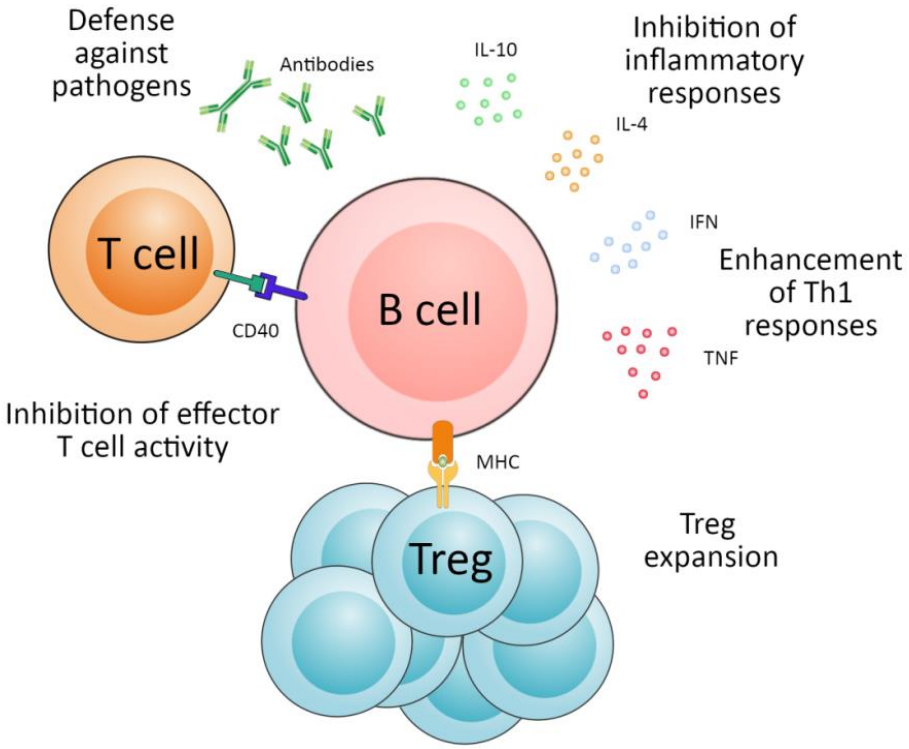


Figure 5. Functional diversity of B cells in Crohn's disease. IFN: interferon; IL: Interleukin; MHC: major histocompatibility complex; Th: T helper cell; TNF: tumor necrosis factor; Treg: regulatory T cell.

In the gut, B cells follow a distinct differentiation pathway and are specialized in IgA production as differentiated plasma cells. Within the gut lumen, secretory IgA interacts with intestinal antigens including the microbiota, food antigens and self-antigens. This system is tightly integrated with both innate and adaptive immune mechanisms, contributing to intestinal immune homeostasis (Figure 5).

The normal IgA immune response at the mucosal sites is skewed toward IgG in the chronically inflamed mucosa of Crohn's disease. The frequent presence of several types of circulating antibodies reactive to microbial antigens and self-antigens (anti-neutrophil cytoplasmic antibody (ANCA)) supports the notion that aberrant immune responses to commensal microorganisms and the host represent a fundamental pathogenic event. The antibodies detected in Crohn's disease patients include anti-*Saccharomyces cerevisiae* antibodies (ASCA), anti-mannobioside

carbohydrate antibodies (AMCA), and anti- predicted lipoprotein from *Escherichia coli* (YidX), among others.[36, 37, 47] The isolated cells from the inflamed mucosa have been shown to secrete antibodies against *Escherichia coli* strains.[46]

Under inflammatory conditions, both human and murine B cells can produce a spectrum of cytokines; including IL-4, IFN-ɣ, IL-2, TNF-α, and IL-12p70.[45, 48] Studies in TCRαKO mice have identified a B cell subset that regulates inflammation by the production of the regulatory cytokine IL-10, known as regulatory B cells (Bregs).[49, 50] In a human blood study, Oka and colleagues found that IL-10–producing Bregs are significantly reduced in frequency in patients with Crohn's disease compared to healthy controls.[51] Nevertheless, there is insufficient evidence to confirm whether Breg reduction or dysfunction directly correlates with the development and pathogenesis of Crohn's disease.

Overall, B cells form a non-negligible component of the mucosal immune system, contributing to epithelial barrier function, the regulation of microbiota diversity, and the development of adequate immune response to both commensal microbiota and food antigens. Nevertheless, most of the studies on the role of B cells in Crohn's disease have focused on their diagnostic or prognostic utility rather than their role in disease pathogenesis. Hence, the role that B cells play in the development of Crohn's disease has yet to be fully elucidated.

T cells

Similarly to B cells, T cells are derived from a lymphoid precursor in the bone marrow. However, in contrast to B cells, T cells do not complete their maturation in the bone marrow. Instead, these cells migrate to the thymus and colonize it. The developing progenitors within the thymus, known as thymocytes, undergo a set of maturation steps. During this process, they must assemble a functional gene for each T cell receptor (TCR) chain. The receptor is assembled in stages, undergoing strict selection processes. [52]

The assembly of TCR genes (*TCRA*, *TCRB*, *TCRG* and *TCRD* – shown in Figure 6) produces a diverse T cell repertoire and is an essential component of thymocyte development. The assembly process for B cell receptor (BCR) genes is very similar. Complementarity-determining regions (CDRs) are the most variable part of both TCRs and immunoglobulins, determining the specificities generated by lymphocytes. CDR1 and CDR2 loops of the receptors contact the major histocompatibility complex (MHC) α-helices, whereas the hypervariable CDR3 regions interact mainly with the peptide.

The CDR3 region is assembled through V(D)J recombination, a site specific recombination process directed by the lymphoid-specific recombinase (RAG, composed of RAG1 and RAG2) and DNA repair proteins. RAG proteins create double-strand breaks at recombination signal sequences that flank CDR3 variable (V), diversity (D) and joining (J) gene segments, and these cleavages are subsequently resolved by non-homologous end joining (NHEJ).

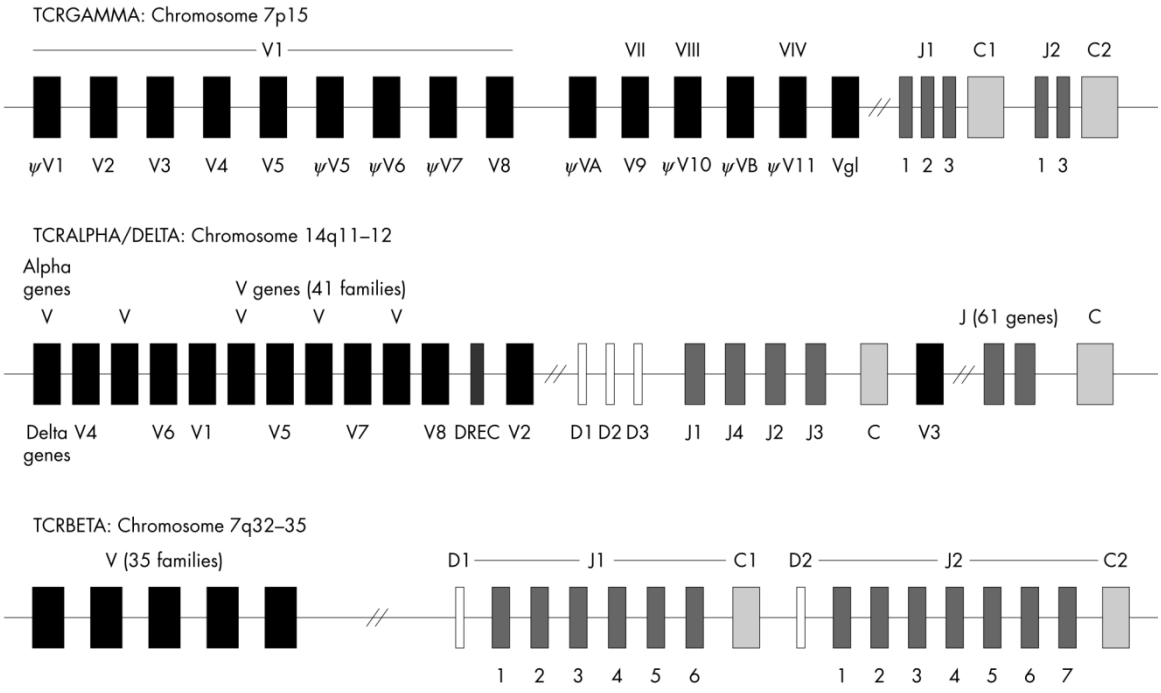


Figure 6. Germline organization of the T cell receptor (TCR) genes. The TCRA, TCRB, and TCRG loci are located on the chromosomes at positions 14q11–12, 7q32–35, and 7p15, respectively. The TCRD loci are also situated on chromosome 14, within the TCRA loci. The shaded boxes indicate the type of gene segment: V, variable (black); D, diversity (white); J, joining (dark grey); C, constant (light grey). Figure from Hodges *et al.*[53]

Unlike typical cellular NHEJ, in which errorless repair is the most positive outcome, error-prone repair in V(D)J recombination is beneficial because it maximizes diversity in the coding sequence of these genes. Patients with mutations in NHEJ genes are unable to produce functional B and T cells and experience severe combined immunodeficiency. Nevertheless, the diversity of the T cell repertoire is not only a consequence of different V(D)J recombinations but also results from nucleotide insertion and deletion at junctional sites, thereby increasing the variety of the antigens recognized.[53]

Development of thymocytes and the subsequent acquisition of the specific TCR is a highly regulated process with well-defined stages (Figure 7). The earliest developing thymocytes lack expression of the co-receptors CD4 and CD8 and are termed double-negative (DN) cells. The DN population can be further sub-divided by the expression of CD25, which is the IL-2 receptor α chain, and CD44, an adhesion molecule.[52] Recombination of *TCRB*, *TCRG* and *TCRD* occur during the double-negative DN2 and DN3 stages of thymocyte development, during the first period of RAG expression. As a result, thymocytes commit to the αβ or γδ lineage (Figure 7).[54]

γδ lineage

The majority of T cells that develop in the thymus commit to αβ T cells. However, approximately 5% of peripheral blood T cells carry the γδ TCR. This sets them apart from the classical and much better known $CD4^+$ and $CD8^+$ T cells that express αβ TCRs. In the gut, γδ T cells are more abundant than in periphery (25–60%). They are present in gut associated lymphoid tissue, but they can also be found as intraepithelial limphocytes.

The selection mechanism of γδ T cells is still largely unknown. Importantly, for unidentified reasons, the expression of particular TCR Vγ and Vδ regions is associated with preferential tissue locations. Hence, the major human peripheral γδ T cell subset (up to 95% in frequency) in healthy adults express a heterodimeric TCR composed of the Vδ2 (DV102S1) and Vγ9 (GV2S1) region chains. By contrast, the Vδ1 and Vδ3 subsets are mainly detected in the thymus, spleen and intestine.[55]

The majority of γδ T cells are activated in an MHC-independent manner, in contrast to MHC-restricted αβ T cells. The antigens of γδ T cells have not been completely identified. It is known that human Vγ9Vδ2 T cells show TCR-dependent activation by certain phosphorylated metabolites such as microbial (E)-4-hydroxy-3-methyl-but-2-enylpyrophosphate (HMB-PP), which is an essential component of some pathogenic bacteria, or eukaryotic isopentenyl pyrophosphate (IPP), which accumulates in tumor cells.[56]

γδ T cells can play a meaningful role in cellular stress surveillance, defending the host against a broad range of pathogens, controlling inflamation and maintaining tolerance towards self-antigens.[55] Other functions involve antigen presentation, promotion of antibody production through B cell interactions and dendritic cell maturation.[57] Stress surveillance is thought to depend on co-stimulatory signals from, for instance, NK-type receptors, in addition to TCR stimuli.[58] Activated human peripheral blood γδ T cells can express high levels of IFN-γ, TNFα and granzymes. Distinct subsets of peripheral γδ T cells, as well as those in the dermis and intestinal lamina propria, are known to produce IL-17. $CD4^+$ γδ T cell clones that produce IL-4 have also been described.[57]

A number of chronic inflammatory diseases are associated with perturbed γδ T cell distributions, such as systemic sclerosis and rheumatoid arthritis.[59] Higher percentages of γδ T cells have been found in the blood of Crohn's disease patients with active disease.[60] Its worth noting that treatment with infliximab, an anti-tumor necrosis factor α (anti-TNF) agent, resulted in the clonal expansion of γδ T cells in the peripheral blood of Crohn's disease patients.[61] These results not only indicate that the observed disturbance may not be an inherent immune anomaly of the disease, but also highlights the potential in vivo role of TNFα signaling in γδ T cell modulation.

αβ lineage

As mentioned above, lymphocyte progenitors migrate from the bone marrow to the thymus, where they undergo a series of developing steps. This process begins in the cortex of the thymus (Figure 7). During the double-negative stage 2, in the first period of RAG expression, thymocytes commit to the αβ or γδ lineage.

Double-negative cells committed to the ab lineage with low expression of CD44 but significant expression of CD25, undergo a process called β-selection. This process selects those T cells that have successfully rearranged their β chain. Unsuccessful cells die by apoptosis. The β chain then pairs with the precursor of its surrogate chain, TCR-α, and produces a pre-TCR. The receptor forms a complex with CD3 molecules, which leads to the survival of the clone and its proliferation. Furthermore, it stops further TCRB rearrangement and induces further differentiation via the up-regulation of CD4 and CD8. The resulting cells are known as double-positive (DP).[62]

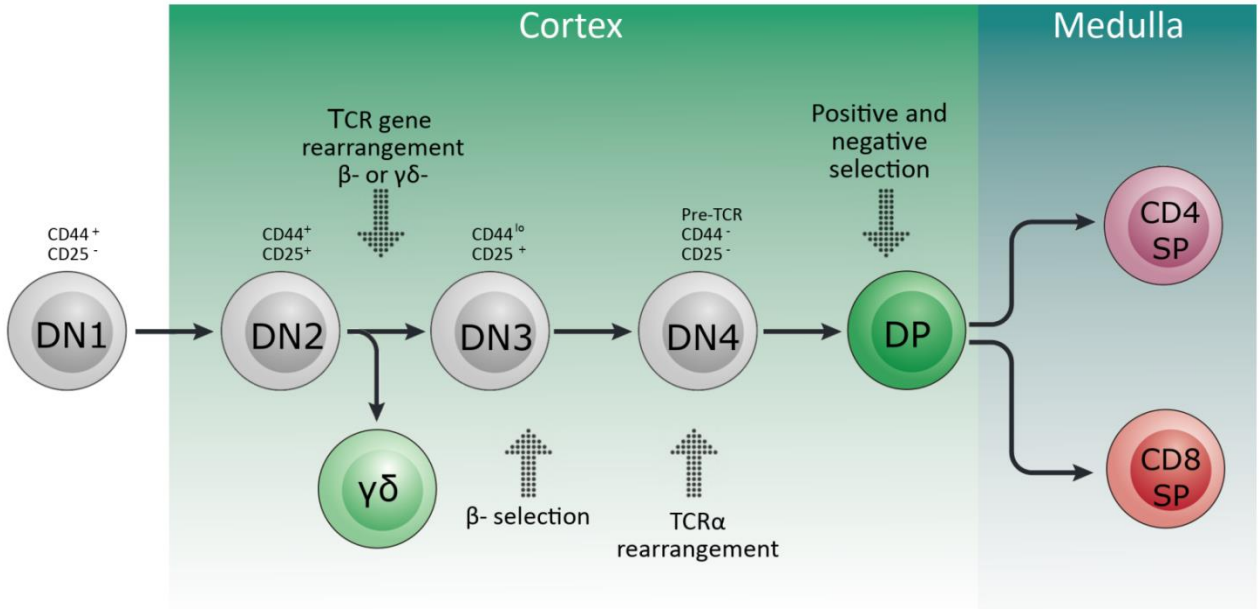


Figure 7. T cell development in the thymus. DN: double negative; DP: double positive; SP: single positive. TCR: T cell receptor. Adapted from Zuniga-Pflucker *et al.*[52]

DP cells re-express the RAG genes that initiate TCRα recombination, thus producing the αβ-TCR. These cells then undergo positive selection in the thymus cortex. DP cells interact with self-antigens in the context of MHC class I or class II molecules. DP cells that join the MHC with adequate strength survive, whereas cells with weaker affinity die by apoptosis.[62] Surviving thymocytes migrate into the thymus medulla to undergo negative selection. During this process, they are presented self-antigens on APCs. Thymocytes with excessive affinity undergo apoptosis. Following selection, down-regulation of either co-receptor produces either naïve CD4 or CD8 single-positive cells that abandon the thymus and circulate into the periphery.[54, 62]

The essential and most studied of the co-receptors on T cells are CD4 and CD8. Both are trans-membrane proteins with extracellular Ig-like domains. Similarly to TCR, they recognize MHC proteins, although they bind to invariable parts of the complex, distant from the peptide-binding region. CD8 binds to class I MHC proteins and is expressed on cytotoxic T cells, whereas CD4 binds to class II MHC proteins and is expressed on helper T cells.[63]

CD8 T cells

As previously stated, $CD8^+$ T cells recognize peptides that are presented through MHC Class I molecules. Importantly, MHC class I molecules are found on virtually all nucleated cells. Hence, these T cells monitor all of the body's cells, ready to destroy any cell considered to be a threat to the health of the host.[64] The responsibility includes defenses against intracellular pathogens, such as viruses and bacteria, and tumoral cells. This protection is mostly fulfilled by the best-characterized subpopulation of $CD8^+$ T cells, cytotoxic T cells. When a cytotoxic T cell recognizes its antigen and becomes activated, it has three major mechanisms to kill the target cell. The first is secretion of cytokines, primarily TNF and IFN-γ.[65, 66] These cytokines further accelerate the innate and adaptive immune response, producing anti-tumoral, anti-viral and anti-microbial effects.[67]

The second major mechanism involves the calcium-dependent release of specialized granules upon recognition of antigen.[64] These granules are modified lysosomes containing two distinct classes of cytotoxic proteins that are expressed selectively on $CD8^+$ T cells. Perforin is one of these cytotoxic proteins, and it polymerizes to form pores in the membrane of infected or malignant cells. This pore allows granzymes (for granule enzyme), another class of cytotoxic protein, to enter into target cells. Once inside the cell, granzymes activate apoptotic cascades.

The third mechanism $CD8^+$ T cell destruction of infected cells is via Fas/FasL interactions. Activated $CD8^+$ T cells express FasL on the cell surface, which binds to its receptor, Fas, on the surface of the target cell.[66] On the cell surface, Fas ligation results in the activation of the caspase cascade, which ends in the apoptosis of the target cell. This mechanism has also been described for $CD4^+$ effector Th1 cells. $CD8^+$ T cells can express both molecules, and Fas/FasL interactions have been described as a mechanism by which $CD8^+$ T cells kill each other to eliminate immune effector cells during the contraction phase at the end of an immune response.[64, 66]

CD4 T cells

$CD4^+$ cells are T cells that express the co-receptor CD4. These cells recognize processed peptides in the vesicles, which are bound to MHC class II molecules. This complex is expressed in APCs, like dendritic cells and macrophages, as well as in B cells. Naïve T cells are prone to become effector T cells the first time they encounter their specific antigen.

Effector T cells activate macrophages and B cell responses to antigen. Naïve $CD4^+$ T cells can differentiate into different types of effector T cells, and this differentiation is driven by the cytokines that APCs secrete when encountering a pathogen. The types of functionally distinct subclasses of effector helper T cells can be distinguished by the cytokines they secrete (Figure 8). However, it must be recalled that the collaborating T cells have plasticity, allowing them to transform into another T cell type according to the micro-environment where they are found once they differentiated.

This differentiation must be highly regulated, since abnormal development of such a subpopulation may eventually lead to an exacerbated or inadequate immune response.[68]

Th1 cells are induced mainly by antigen-presenting cells that harbor pathogens in intracellular vesicles. In turn, these cells activate macrophages and induce B cells to make IgG antibodies. This class of antibody is very effective at opsonizing pathogens for elimination by phagocytes.[63] Th1 also activate dendritic cells, which increase their expression of class II MHC proteins and various cytokines, particularly IL-12. This interleukin stimulates T cells to differentiate into Th1. This generates a positive feedback loop that increases the number of Th1 cells and their activation by dendritic cells, resulting in the activation of more macrophages.[64, 65]

Th2 cells are induced mainly by IL-4 cytokine secreted by the APC, which at the same time induces the activation of GATA3 transcription factor. They produce IL-4, IL-5, IL-10, and IL-13 and its primary host defense will target extracellular pathogens. A Th2 cell can stimulate B cells to produce most classes of antibodies, including IgE, IgA and some subclasses of IgG antibodies, that bind to basophils, mast cells and eosinophils.[63] These cells release local mediators, like prostaglandin, that can cause coughing or diarrhea and thereby help expulse extracellular pathogens and larger parasites from epithelial surfaces of the body.[65]

A mouse model of multiple sclerosis provided the first clues to the possibility that other T cell effector functions, beyond those attributed to the Th1 and Th2 subsets, could be contributing to the onset and progression of autoimmune disorders.[69] This led to the theory that there exists a new subset of T-helper cells different from the classical Th1/Th2 paradigm. This novel subset, named **Th17** for its production of IL-17, involves a combination of cytokines, transcription factors, and immunological functions that make it distinct from both Th1 and Th2 cells. The Th17 lineage provides a unique mechanism for protection against bacterial and fungal pathogens via the production and induction of inflammatory cytokines and other proteins.[70]

Another CD4⁺ T cell lineage, regulatory T (**Treg**) cells, are critical in the prevention of immune responses directed against self-antigens.[71, 72] Highly auto-reactive CD4⁺ T cells are eliminated from the medulla of the thymus during negative selection. Nevertheless, a portion of them with intermediate auto-reactivity differentiate into Treg cells in a process known as agonistic selection.[54] These cells are characterized by the expression of Foxp3 and are specialized in the control of peripheral immune tolerance. Foxp3-positive Treg cells are largely distributed in the thymic medulla and migrate to peripheral tissues. There are two subsets of Tregs, natural thymus-derived (nTregs) and induced peripheral-derived (iTregs) Treg cells.[71] Secondary lymphoid organ regulatory cells are mostly nTreg cells, which are required for maintaining immune tolerance. On the other side, iTreg cells can powerfully control immunity at sites of inflammation, especially at mucosal surfaces.[71]

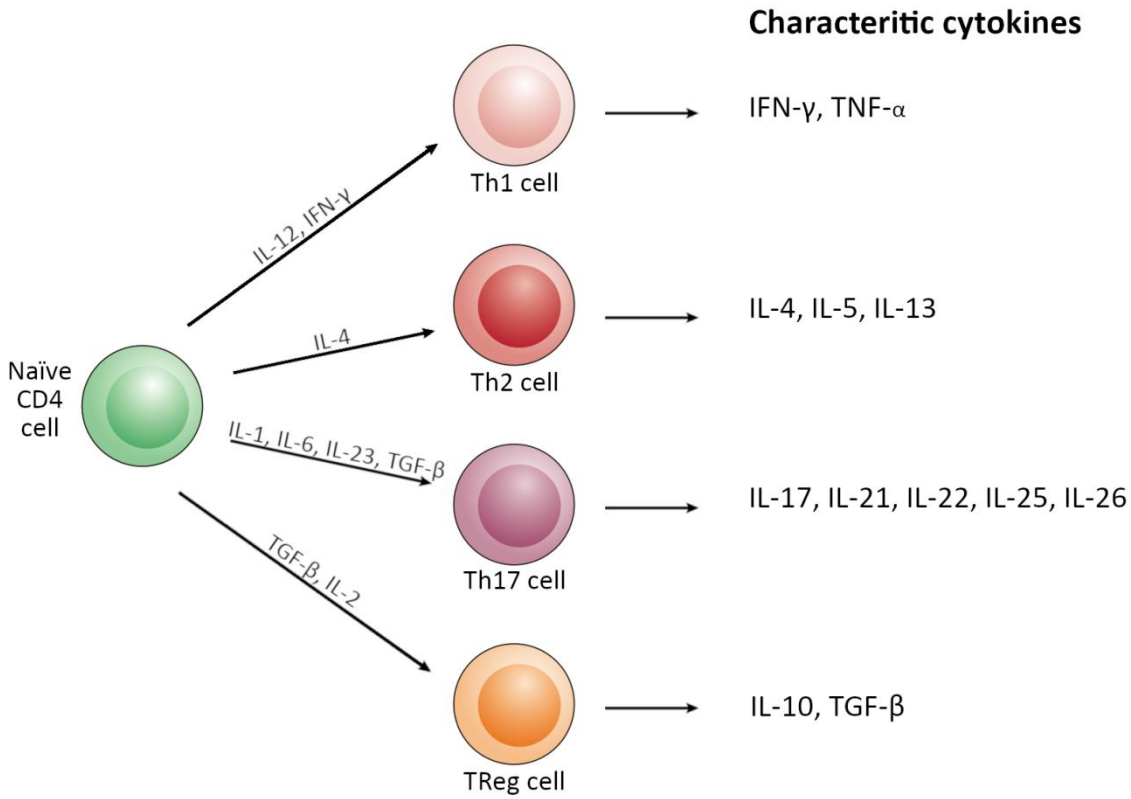


Figure 8. Helper T cell differentiation. Classical view of the differentiation of the T cell. IFN-γ: interferon-γ; IL: interleukin, TGF-β: Transforming growth factor β; TNF: tumor necrosis factor. Adapted from O'Shea *et al.*[68]

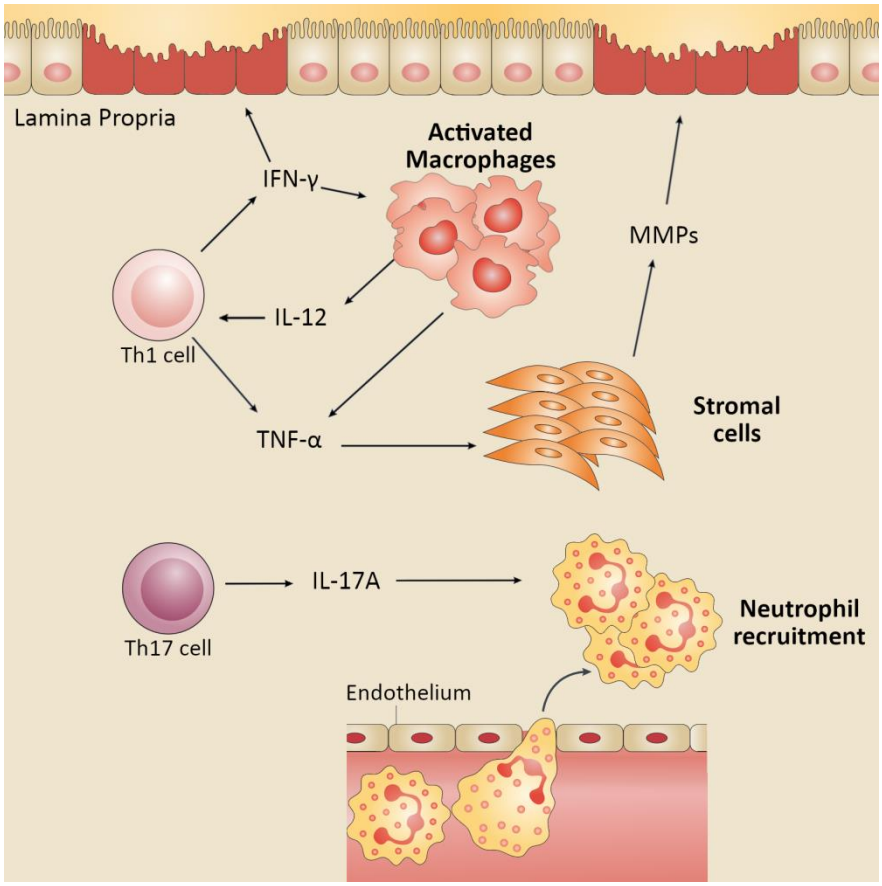


Figure 9. Adaptive T cell responses in gut in Crohn's disease. IL: interleukin; IFN: interferon; MMPs: matrix metalloproteinases; Th: T helper; TNF: tumor necrosis factor. Adapted from Geremia *et al.*[43]

In an inflammatory context, naïve T cells differentiate into effector T-helper Th1 and/or Th17 cells (Figure 9). Th1 cells are induced by IL-12 and IL-18 and produce vast amounts of IFN-γ and TNF-α, both of which trigger the apoptosis of epithelial cells.[73] IFN-γ activates intestinal macrophages, which in turn produce additional TNF-α. This cytokine promotes the differentiation of lamina propria stromal cells into activated myofibroblasts, which produce substantial amounts of matrix metalloproteinases.

These large amounts of matrix metalloproteinases degrade both the extracellular matrix and the basement membrane, thus inducing enterocyte apoptosis.[73] On the other hand, Th17 cells secrete IL-17A, a cytokine that induces the recruitment of neutrophils to sites of active inflammation. IL-17 is also known to induce the production of proinflammatory cytokines (such as TNF-α, IL-1B and IL-6) and matrix metalloproteinases.[74]

Furthermore, both Th1 cell-associated surface receptors (such as IL-2Rβ2) and Th1 transcription factors (such as STAT4 and T-bet) are known to be expressed by lamina propria T cells in patients with Crohn's Disease. Additionally, mucosal T cells from Crohn's disease patients have been shown to secrete higher amounts of IFN-γ and IL-2 than healthy individuals.[73, 75]

Nevertheless, in order to cause damage in the gut, T cells must enter into the intestinal mucosa. The success of this process depends on the recognition between adhesion receptors expressed on the T cell surface and their ligands on the gut vascular endothelial surface, as well as in chemokine/chemokine receptors. Integrins are cell-surface glycoproteins formed by 2 chains, α and β, which can form approximately 24 unique proteins. α4β7, a well-characterized gut-homing receptor on T cells and B cells, specifically binds to MAdCAM-1 on the intestinal vasculature. Thus, α4β7 serves as a primary mediator of inflammation in the gastrointestinal tract. Blocking the interaction between receptors and their ligands on endothelial cells can impair the recruitment of certain T cell subsets into the gut. This fact has led to the development of biological therapies for Crohn's disease that interfere with T cell homing to the gut.[3, 74]

Resident Memory T cells

Once in the intestinal tissue, memory T cells terminally differentiate and become resident. This resident-memory T cells are a recently identified lymphocyte lineage that persists in tissues and provides protective immunity under healthy conditions.[76] These cells express tissue-retention proteins. In particular, resident memory T cells are known to express the integrins αE (CD103) and α1 (ITGA1). Moreover, nearly all resident-memory T cells express C-type lectin CD69, which prevents cells from leaving the tissue and antagonizes the lymph node exiting-sensitizing protein S1PR1. In the same line, they have low expression of S1PR1 gene itself, and lymph node homing markers as CD62L and CCR7. [76]

Resident-memory T cells are the most abundant memory T cells in the intestinal mucosa in healthy humans.[77] Dysregulation of the these T cells may contribute to inflammatory and autoimmune diseases.[77] Their role in Crohn's disease is not completely understood. A recent study from Bishu *et al.* observed an expansion of resident-memory $CD4^+$ T cells in active colonic tissue from Crohn's disease patients (n=23) as compared to non-IBD controls (n=10).[78]

Chapter 3: treatments for Crohn's disease

Currently, the goal of Crohn's disease treatment is not limited to clinical remission, but rather to achieve mucosal healing, which means the reconstitution of the intestinal epithelial barrier structure and function. Mucosal healing is predictive of sustained clinical remission, lower hospitalisation rates and resection-free survival. Moreover, it should be regarded as an initial event in the regulation of the deeper layers of the inflammed bowel wall, instead of as a complete healing of the gut.[79] To date, knowledge about Crohn's disease etiology remains insufficient, and all the available treatments are focused on blocking the inflammatory cascade. The management of a patient with Crohn's disease should take into account the disease activity, location and behaviour, and should always be discussed with the patient.[80]

Standard Therapy

The standard therapy for Crohn's disease includes the use of corticosteroids, immunomodulators, biological agents and surgery. Though not a first-line treatment, **antibiotics** are considered appropriate for septic complications (sepsis or abscess), symptoms attributable to bacterial overgrowth (such as bloating or flatulence), or perianal disease. They are also used prophilactically in acutely active Crohn's disease in the setting of toxic megacolon to treat the translocation of bacterial toxins.[81]

Steroids are able to induce clinical remission in luminal Crohn's disease.[80] They are among the least expensive agents that can be prescribed and they work quickly. Nevertheless, corticosteroids are unable to maintain remission or achieve mucosal healing. Additionally, they have frequent and severe adverse effects, including higher rates of opportunistic infections.[81, 82]

Immunomodulators have shown their efficacy mainly as a maintenance therapy of remission in Crohn's disease. They were in use for other immune diseases long before they began to be routinely studied or prescribed for Crohn's disease. Thiopurines (such as azathioprine or 6-mercaptopurine) and methotrexate have

been proven to maintain remission in Crohn's Disease. The early use of these modulators in combination with corticosteroids is an appropriate option in moderately active localized ileocecal Crohn's disease. Adverse effects include gastrointestinal toxicity (nausea, vomiting and diarrhea) but the principal long-term concerns are hepatotoxicity and pneumonitis.[80, 81]

Among **biologic agents**, anti-TNF agents have been widely used for the treatment of Crohn's disease. Adalimumab (Humira®) and Infliximab (Remicade®) are IgG1 anti-TNF monoclonal antibodies with potent anti-inflammatory effects. Their efficacy is due, at least partly, to apoptosis modulation in lamina propria mononuclear cells.[83] Certolizumab pegol (Cimzia®) is a monoclonal anti-TNF Fab-antibody fragment with proven clinical efficacy despite the lack of pro-apoptotic effects.[84]

All approved anti-TNF agents are antibodies that block the pro-inflammatory cytokine TNFα, which is clearly involved in the pathogenesis of Crohn's disease, as previously mentioned. Numerous controlled trials have demonstrated the efficacy of these anti-TNF agents for active inflammatory Crohn's disease.[85, 86, 87] They have been approved for both induction and maintenance therapy. Some adverse events associated with anti-TNF treatment include lymphoma, non-melanoma skin cancer and some complex infections, such as those secondary to mycobacteria and fungi.[81] All currently available anti-TNF therapies have similar adverse events and efficacy profiles for inflammatory Crohn's disease. Therefore, the choice between them depends on availability, route of delivery, patient preference, cost and national guidelines. As an example, infliximab and adalimumab are currently approved for use in Crohn's disease in many countries, while certolizumab pegol is not approved in the European Union.[80] Figure 10 schematically depicts the mechanism of action of biologic agents for the treatment of Crohn's disease.

The "*step-up*" strategy is the traditional approach used in Crohn's disease patients. It consists of the sequential introduction of the aforementioned drug groups according to the severity and anatomical distribution of the disease. For example, in patients with moderate to severe disease treatment would begin with steroids, followed by immunomodulators. Anti-TNF agents would be only

considered when there is objective evidence of active disease that was previously steroid-refractory, steroid-dependent, or steroid-intolerant. Similar to other immune-mediated diseases such as rheumatoid arthritis, Crohn's disease is characterized by cumulative tissue damage over time.

The "*top-down*" strategy implies the introduction of early combined immune-suppression (anti-TNF agents in tandem with immunomodulators) from the first disease flare-up. This strategy is based on the hypothesis that an early introduction of anti-TNF agents in high-risk patients can change the disease course, thus avoiding its progression and further tissue damage. Data from the Study of Biologic and Immunomodulator Naïve Patients in Crohn's Disease (SONIC) Trial confirmed the superiority of combined immunosuppression in achieving mucosal healing in early Crohn's disease.[88] After achieving remission, the doses of corticosteroids should gradually be decreased.[80]

Surgery is not curative for Crohn's disease. Nevertheless, there are circumstances in which it should be considered as an option, such as in localized Crohn's disease unresponsive to biologics. In such cases it should be discussed at an early stage of the disease. Based on a systematic meta-analysis of 30 population-based studies, the cumulative incidence of abdominal surgery in Crohn's disease is 16% at 1 year after diagnosis, 33% at 5 years, and 47% at 10 years.[89] It has been observed that over the past several decades the risk of surgery has significantly decreased in patients with Crohn's disease. This decrease in the risk of surgery could be due to broader medical education and subsequent earlier diagnosis of the disease, to a more frequently practiced multidisciplinary approach to the patient, and to earlier and more aggressive medical treatments.

However, only a minority of patients will present sustained clinical remission with the currently available therapies. As surgery will not be an option in most cases, the need for new therapeutic alternatives has led to the development of new treatment molecules and approches, including cell therapies. [84, 85, 86, 87, 90]

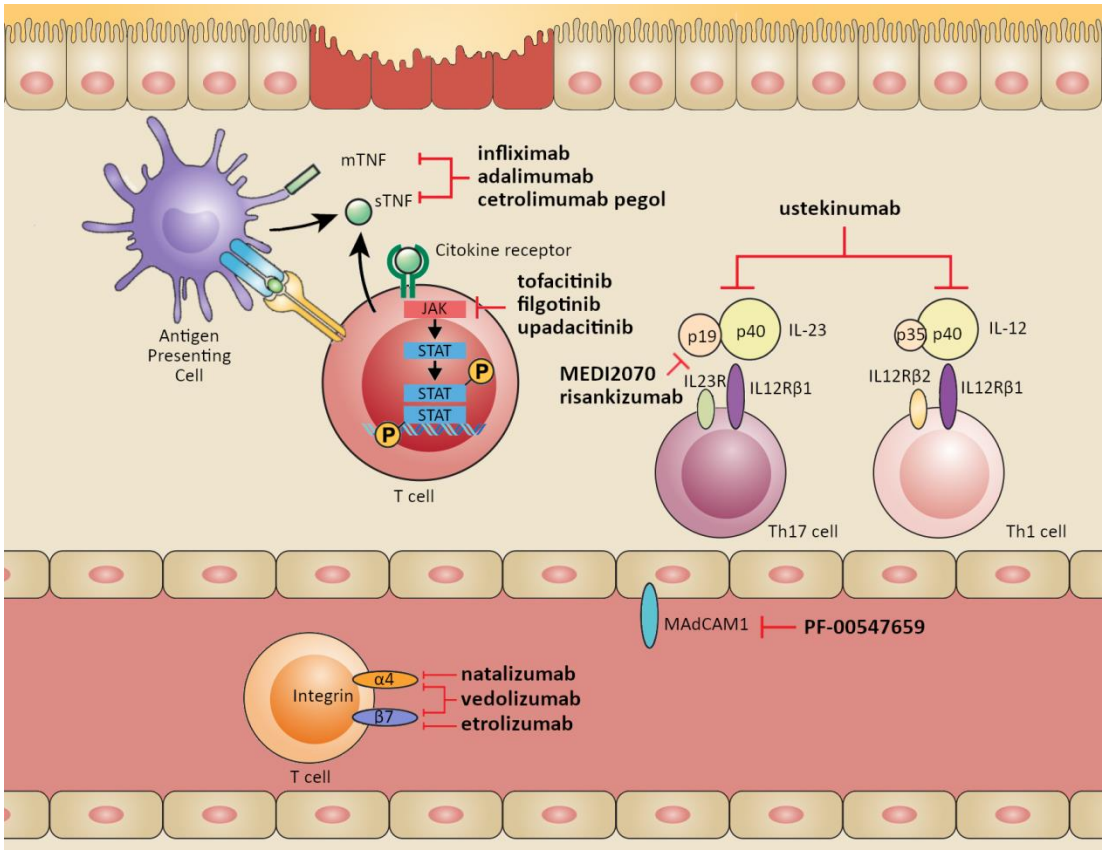


Figure 10. Biologic agents for the treatment of Crohn's Disease: standard and new treatments. Schematic representation of the mechanism of action of different biologic agents. Adapted from Kim *et al.*[99]

New treatments for Crohn's disease

Anti-adhesion molecules

Integrins comprise a family of heterodimeric transmembrane receptors that are constitutively expressed on leucocyte surfaces and are activated by pro-inflammatory cytokines. As mentioned above, integrins are formed by 2 chains, α and β, and integrin α4β7 is a well-characterized gut-homing receptor on T cells and B cells. Blocking the interaction between the receptors and their ligands on endothelial cells prevents lymphocytes from entering the gut. This mechanism is the basis for the development of biological therapies for Crohn's disease.

Natalizumab, an anti-α4 integrin IgG4, was the first anti-integrin agent used in Crohn's disease patients. α4 is also expressed as α4β1, a non-gut selective integrin. Natalizumab has proven its effectiveness in the induction and maintenance of remission in Crohn's disease.[91] However, it prevents leukocytes from entering the central nervous system and the gut. In fact, by reducing cell trafficking to the brain, natalizumab has been reported to affect cerebral antiviral immunity. In some cases, it can cause a fatal brain infection known as progressive multifocal leukoencephalopathy due to reactivation of the John Cunningham virus.[92, 93] Hence, its use has been significantly limited and it is not approved for Crohn's disease treatment in Europe.

Vedolizumab is an anti-α4β7 integrin humanized IgG1 that inhibits the adhesion of T and B cells to the gastrointestinal tract. It has shown efficacy as an induction and maintenance treatment for Crohn's disease.[94] Because vedolizumab acts selectively on the intestine, it causes almost negligible systemic immunosuppression, and thus adverse effects are minor. In the phase 3 GEMINI 2 trial, the most commonly reported adverse reactions to vedolizumab were nasopharingitis, arthralgia, headache and nausea.[94] Vedolizumab has been approved for the treatment of moderately to severely active Crohn's disease in patients who have had an inadequate response, lost response, or were intolerant to either conventional therapy or to anti-TNF treatment.

Anti-adhesion molecules under investigation

Etrolizumab is a humanized monoclonal antibody that acts against the β7-subunit of the integrins α4β7 and αEβ7. The αEβ7 integrin binds selectively to E-cadherin on epithelial cells and is expressed by mucosal T lymphocytes. It may play a role in the retention of T cells in mucosal tissue.[95] Phase III studies examining the efficacy of etrolizumab in Crohn's disease are currently underway.[96] **AMG-181** is a fully human monoclonal antibody that, like vedolizumab, blocks the binding of α4β7/MadCAM-1. Scant data is available, but no adverse events have been reported and trials are currently undergoing for IBD, both ulcerative colitis and Crohn's disease.[97]

One gut-selective anti-adhesion molecule strategy involves inhibiting MadCAM-1, the ligand of α4β7 integrin. The fully human IgG2 anti-MadCAM-1 antibody, **PF-00547659**, has been tested in Crohn's disease, but the clinical differences between PF-00547659 and a placebo did not reach statistical significance in patients with moderate-to-severe Crohn's disease.[98]

Anti-IL-12/IL-23 agents

Ustekinumab is a fully humanized monoclonal IgG antibody that targets the p40 subunits of IL-12 and IL-23 (Figure 10). Both are pro-inflammatory cytokines that interact with the differentiation and proliferation of Th1 and Th17 cell subsets respectively.[99] The CERTIFI studies showed the efficacy of ustekinumab in inducing and maintaining remission in patients with moderately to severely active Crohn's disease that has previously been refractory to either anti-TNF agents or other common theraphies.[100] A recent study provides evidence for the efficacy of ustekinumab in inducing endoscopic healing.[101]

MEDI2070 is a fully human IgG2 monoclonal antibody, which selectively binds the p19 subunit of IL-23. A phase IIa induction study demonstrated the clinical efficacy of MEDI2070 in 121 patients with moderate-to-severe Crohn's disease, who previously had an inadequate response to anti-TNF therapy.[102] Similarly to MEDI2070, **risankizumab** potently binds to p19 and prevents its binding to the IL-23 receptor. The results of a phase II trial in moderately-to-severely active Crohn's disease were favourable.[103] The majority of enrolled patients had been exposed to anti-TNF before (94%), and it was a largely refractory population that had shown primary non-response (30%) or secondary loss-of-response (28%) to treatment. It was observed that risankizumab was able to induce endoscopic remission at week 12. Furthermore, it showed a favorable safety profile, with fewer severe adverse events reported compared to the placebo. Although ustekinumab and risankizumab have not yet been compared in parallel in Crohn's disease, a phase II randomized-trial in patients with psoriasis showed a link between risankizumab and clinical responses superior to those associated with ustekinumab.[104] Nevertheless, the trial was not large or long enough to draw any conclusions about safety.

Janus kinase inhibitors

Janus kinases (JAK) are a family of intracellular protein tyrosine kinases that are crucial to the signaling of immune receptors. A number of cytokines execute their function by activating JAK-dependent pathways, thus playing an important role in mounting immune responses and in developing immune-mediated disorders. The inhibition of JAKs can result in the suppression of B and T cells.[105] Therefore, the JAK family is an important target for Crohn's disease.

Tofacitinib is an oral JAK-1 and JAK-3 inhibitor that blocks JAK-dependent intracellular signaling pathways. This drug has not shown efficacy in inducing a clinical response in Crohn's disease patients who have failed conventional therapy. This is despite the fact that tofacitinib has been approved for rheumatoid arthritis[106] and has shown promising effects in ulcerative colitis.[107] **Filgotinib** is an oral JAK-1 inhibitor. In a randomized, double-blinded phase II trial (n = 174), filgotinib was effective in inducing clinical remission, although it failed to produce any endoscopic improvements.[108] Results from a phase 3 study have not yet been published. Another drug under development is **upadacitinib**, a JAK1 inhibitor that has shown promising results in Crohn's disease patients. [109]

Nevertheless, the use of JAK inhibitors in Crohn's disease has led to reports of adverse events; specifically, increases in opportunistic infections and anaemia.[105, 110] Consequently, their use may best be limited as stand-alone treatment, and not one to be administered in conjunction with other immunosuppressives. In addition, costicosteroids should be discontinued as soon as clinically possible if JAK inhibitors are to be prescribed.

Stem cell therapy for Crohn's disease

Two types of multipotent stem cells are currently used for the treatment of Crohn's disease: mesenchymal stem cells (MSCs) and hematopoietic stem cells (HSCs). Both types are found in the bone marrow, although MSCs can also be obtained from different tissues (e.g., adipose tissue, umbilical cord and placenta). Since HSCs transplantation (HSCT) is the main treatment type examined in this thesis, the next chapter is completely dedicated to it.

Mesenchymal stem cell transplantation

Mesenchymal stem cells are multipotent cells that can differentiate into a variety of cell types; including osteoblasts, myocytes, chondrocytes and adipocytes. MSCs can be isolated from the bone marrow, connective tissue, adipose tissue, umbilical cord and placenta. Once MSCs are isolated, they can be expanded *ex vivo* for clinical use. MSCs contribute to the creation of niches in different tissues, including the intestine, and their immunomodulatory properties make them capable of altering those cells involved in the immune response. Their lack of MHC-II and co-stimulatory molecule expression makes them less immunogenic, allowing allogeneic transplantation without need of donor-recipient matching or bone marrow conditioning. In Crohn's disease patients, autologous and allogenic MSCs have been locally applied for the treatment of perianal fistula, in order to achieve fistula tract closure, in several phase I and II studies, with no safety concerns and positive clinical outcomes.[111, 112, 113, 114, 115, 116]

Alofisel (Cx601), which consists of adipose-tissue derived allogeneic mesenchymal stem cells, has proven to be effective at inducing and maintaining fistula closure when applied locally, close to the tract, in conjunction with surgical preparation of the fistula track.[117, 118] The drug received approval in Europe and a second phase III trial is being conducted.

Chapter 4: autologous hematopoietic stem cell transplantation as treatment for Crohn's disease

Stem cell transplantation is an accepted therapy for hematologic disorders, aplastic anemia and immunodeficiencies. In the context of autoimmune diseases, the serendipitous benefits of transplantation were initially reported in patients suffering from both immune-mediated diseases (IMDs) and hematologic disorders.[119, 120]

Therefore, in 1997 the European Group for Blood and Marrow Transplantation (EBMT) launched the Autoimmune Disease Working Party (ADWP).[121] Since then, autologous HSCT has demonstrated its efficacy for the treatment of different IMDs such as multiple sclerosis, severe systemic sclerosis, systemic lupus erythematosus, juvenile chronic arthritis, immune thrombocytopenia, autoimmune cytopenias and Crohn's disease.[122, 123, 124]

Multicenter randomized clinical trials have also been conducted by the EBMT/ADWP in collaboration with various scientific societies to assess the impact of HSCT. These include ASTIMS trial for multiple sclerosis[125], the ASTIS trial for severe systemic sclerosis[126] and the ASTIC trial for Crohn's disease[127]. Autologous HSCT is the only treatment capable of inducing long-term and drug-free remission in several refractory IMDs.[128] According to the EBMT, autologous HSCT should be considered for patients with severe and progressive IMD despite the availability of standard and/or approved therapies.[124] The aim of this procedure is to eradicate the auto-reactive memory immune response and regenerate a self-tolerant immune system from haematological precursors, which cannot be achieved by conventional therapy.[129]

Stages of autologous HSCT

Despite its apparent benefits, HSCT is associated with higher rates of mortality and severe adverse effects, such as infectious complications and drug toxicity. [130, 131] Patients need to be carefully selected and thoroughly screened before transplantation to determine whether the potential benefits of transplantation outweigh its risks, as well as to be sure that the patient condition is optimal for such treatment. Therefore, autologous HSCT is only offered to a small percentage of patients with refractory and complicated disease, for whom no other therapeutic options are available. In our study, those patients with severe comorbidities, symptoms unrelated to disease inflammatory activity (e.g., stenosis), significant infection risk, severe malnutrition, poor compliance and/or pregnant women were excluded. [130, 131]

Hematopoietic stem cells (HSCs) are collected by apheresis from the peripheral blood after a **mobilization** treatment, which promotes the production and release of these cells from the bone marrow into the systemic circulation. EBMT's recommended mobilization protocol consists of cyclophosphamide at a dose of 2-4g/m^2 followed by granulocyte colony stimulating factor (G-CSF) at a dose of 5-10μg/kg.[132] In our study, mobilization of HSCs was achieved via the use of an intravenous cyclophosphamide dose of 2g/m^2/day administered on 2 consecutive days. Five days after the last cyclophosphamide infusion, subcutaneous G-CSF was started at a dose of 10 mg/kg/day until leukapheresis was completed (Figure 11).[130, 131]

When stem cells are mobilized for collection, the patient's IMD has to be closely monitored to prevent not only flares, a potential consequence of the necessary administration of G-CSF, but also infectious complications stemming from cyclophosphamide treatment.[124, 128, 132] Therefore, patients are hospitalized during the mobilization phase in order to maximize safety. Supportive care measures include hygienic measures (private room, hand washing and limited visitor access) and the use of antimycotic agents among others. Parenteral nutrition is also established during the aplasia periods. [127, 130, 131]

Collected HSCs are cryopreserved until infusion. The amount of HSCs collected, as recommended by EMBT, is a minimum of $2x10^6$ $CD34^+$/kg.[124] In this study, a minimum of $3x10^6$ $CD34^+$/kg had to be obtained to proceed with the conditioning phase and, whenever possible, a backup harvest of at least $2x10^6$ $CD34^+$/kg was performed[130, 131], as recommended by EBMT.[132]

After mobilization, patients enter the **conditioning phase**, which consists of an immunoablative protocol that aims to eliminate auto-reactive T cells from the host and to deplete T cells from the autologous graft. The conditioning protocol used in this study consisted of intravenous cyclophosphamide (total dose of 200 mg/kg (50 mg/kg/day during 4 consecutive days, -5 to -2 days before transplantation)) and *in vivo* T cell depletion step using intravenous 7.5 mg/kg of rabbit anti-thymocyte globulin (ATG) (2.5 mg/kg/day for 3 days, -3 to -1 days before transplantation) prior to administration of harvested HSCs by intravenous transfusion (designated as day 0, Figure 11).[130, 131] ATG is a polyclonal antibody that targets a variety of immune system proteins and that has diverse effects on the immune system: it depletes T cells in blood, induces apoptosis in B cell lineages, modulates dendritic cell functional properties, modulates leukocyte/endothelium interactions, and induces the expansion of regulatory T cells.[133]

Ex vivo T cell depletion can be used in addition, or as an alternative, to *in vivo* ATG, in order to obtain a $CD34^+$-rich graft, mostly via magnetic separation for CD34 selection.[124, 128, 134] The evolution of patients who receive *ex vivo* selected cells is similar to that of patients who received non-selected graft.[135, 136, 137] Nevertheless, Cassinotti *et al.* [138] proposed that *in vivo* T cell depletion without $CD34^+$ cells selection exhibited better tolerance and shorter time to engraftment in Crohn's disease. The ASTIC clinical trial conditioning protocol consisted of *in vivo* administration of rabbit ATG.[127] A recent retrospective analysis from the EBMT showed that the majority of Crohn's disease patients undergoing autologous HSCT outside the ASTIC trial had also been conditioned exclusively by *in vivo* T cell depletion (69/82 patients).[139]

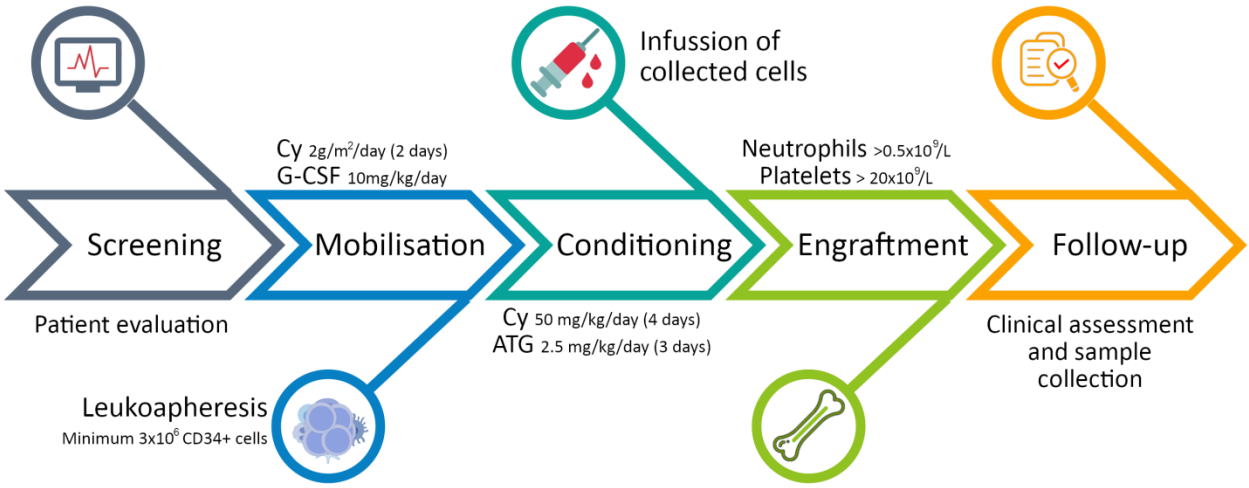


Figure 11. Different stages of the Hematopoietic Stem Cell Transplant protocol. ATG: anti-thymocyte globulin; Cy: cyclophosphamide.

Within the first few days after transplantation, the reinfused HSCs migrate to the bone marrow and begin the process of generating replacement blood cells. **Engraftment** is typically defined as 3 days of neutrophils rising above $0.5x10^9$/L and/or platelets rising above $20x10^9$/L.[140] During the 2 weeks following transplantation the patient should remain under strict supportive care; including the administration of antibiotics (prophylactic and therapeutic), transfusions, symptomatic care, parenteral nutrition and monitoring.[131, 140]

Reconstitution of the immune system after autologous HSCT

As previously mentioned, the rationale behind autologous HSCT is that after the profound depletion of immune cells, not only will a new and naïve immune system be reconstituted from the graft, but immunotolerance will also be re-established through thymus selection of the T cell repertoire. However, exactly how, and to what extent, HSCT achieves these changes to the diseased immune system is still unknown.[128, 141, 142] As yet undetermined, for instance, are which cells need to be depleted and which are necessary to maintain. Indeed, despite the conditioning regimen, not all T cells are completely depleted and potentially pathogenic T-cell clones can still be detected after HSCT in multiple sclerosis.[143]

Lymphopenia induced proliferation of lymphocytes

Reconstitution of the immune system occurs at different rates in the various cell lineages and subsets (Figure 12). Neutrophil recovery is achieved after a median time of 11 days (range 7–17) in a 29 Crohn's disease patient cohort undergoing autologous HSCT in our group.[130, 131] Following engraftment, an initial phase of immune reconstitution was characterized by a significant, though transitory, increase in the proportion of NK cells and residual memory B and T cells. The lymphopenic environment induced by the conditioning phase drove the expansion of these populations via the action of homeostatic cytokines and antigen stimulation. [134, 141, 142, 144, 145]

As previously mentioned, some memory T cells can survive HSCT conditioning, which can hamper the restoration of immune tolerance.[143] Clonal expansion increases the absolute number of T cells, leading to TCR oligoclonality.[143] In a 2005 study[146], patients with autoimmune disease maintained sustained remission after HSCT even though pre-existing dominant T-cell clones remained present after transplantation, suggesting that these cells were unable to induce disease activity within their new environment.

Cytotoxic $CD8^+$ T cells are the first T cell subset to normalize and the ratio of naïve to memory $CD8^+$ T cells remains constant following autologous HSCT.[142] In patients with multiple sclerosis, early expression of the inhibitory molecule programmed cell death-1 protein (PD-1) on $CD8^+$ T cells paralleled good clinical response post-treatment.[141] PD-1 expression is likely to maintain self-tolerance during lymphopenia-induced proliferation.[141, 147]

In contrast, emerging B cells at 3, 6, and 12 months after HSCT are predominately naïve (IgD^+CD27^+) and consequently a significantly lower percentage of memory B cells is observed compared to age-matched controls.[156] In order to protect the transplant recipient against serious vaccine-preventable infections that may occur during the post-transplant period, the EBMT's guidelines recommended vaccination of these patients.[148]

Thymic reactivation

Following lymphoid ablation, the thymus volume enlarges.[149] As explained in Chapter 2, the thymus produces naïve T cells with unique TCRs. The thymus has been reported to be functionally activated following HSCT.[145, 146] Consequently it is responsible for establishing the new TCR repertoire after HSCT. During TCR rearrangements, DNA by-products known as signal-joint T cell receptor excision circles (TRECs) are generated. Early naïve T cells, known as recent thymic emigrants (RTE), can be identified via analysis of TRECs, which can be used to determine thymic function.[145, 146]

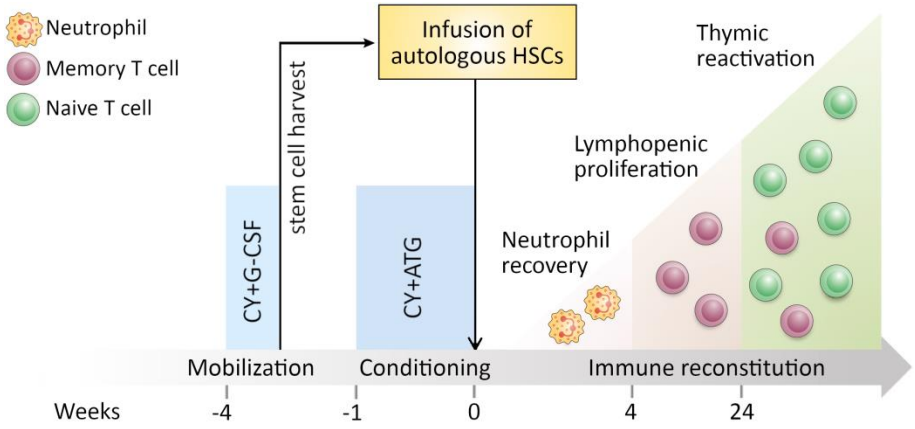


Figure 12. Immune recovery after HSCT in patients with autoimmune disease. ATG: anti-thymocyte globulin; CY: cyclophosphamide; G-CSF: granulocyte colony stimulating factor; HSCs: hematopoietic stem cells. Adapted from Alexander *et al.*[134]

$CD4^+$ T cell reconstitution is more dependent on thymopoiesis than it is on $CD8^+$ cells, and $CD4^+$ cell numbers often require years to normalize.[141] Furthermore, following HSCT the residual naïve T cells disappear, resulting in decreased naïve and increased effector memory T cells during the first 3 months post-transplant.[141] The $CD4^+$ T cell compartment undergoes reshaping post-HSCT compared to baseline. A peripheral blood study of multiple sclerosis found that the most abundant $CD4^+$ TCR clones before HSCT were undetectable after treatment, and patients largely developed a new $CD4^+$ TCR repertoire.[143]

In contrast, dominant $CD8^+$ clones before treatment were still present in the reconstituted repertoire, which was created by the clonal expansion of cells present before treatment. Importantly, patients non-responsive to HSCT in that study had less diversity in their repertoire during the early reconstitution process, reinforcing the notion that repertoire complexity is crucial for the reestablishment of immune tolerance. [143]

Treg cells might also help in restoring self-tolerance. Several human studies involving patients with Crohn's disease, multiple sclerosis, juvenile idiopathic arthritis or systemic lupus erythematosus undergoing HSCT suggest that there is an increase in the Treg subset after HSCT.[136, 150, 151, 152] Clerici and colleagues assessed the impact of autologous HSCT on peripheral blood lymphocytes in a 7-patient cohort of Crohn's disease patients, and demonstrated the restoration of dysregulated T effector cell responses, together with a significant increase in $Foxp3^+$ Treg cells.[136] The one patient in that cohort who relapsed was the only subject who exhibited a clear trend towards a decrease in Treg cells.

Almost all data regarding immune reconstitution following autologous HSCT are based on cells located in the periphery. However, aberrant inflammation in IMDs is primarily located within the tissue's target. For instance, in Crohn's disease the TCR repertoire in affected mucosa is strongly oligoclonal.[129] Following autologous HSCT, mucosal healing of the affected regions does occur[130] but whether this stems from the generation of a polyclonal TCR repertoire in the mucosa remains unknown. Moreover, the extent of tissue penetration of ATG is unclear. In a study performed in ATG-treated non-obese diabetic mice, the depletion of both $CD4^+$ and $CD8^+$ T cells was less efficient in the spleen than in the peripheral blood.[153] It is therefore possible that resident memory T cells in tissues are not completely depleted by ATG.

Autologous HSCT in Crohn's disease

Several studies have published successful results after autologous HSCT in small series of patients with Crohn's disease.[138, 154, 155, 156, 157, 158] As mentioned above, the ASTIC trial assessed the value of HSCT in refractory Crohn's disease.[127] Its protocol was designed to evaluate whether HSCT produced long-term endoscopic and clinical disease remission. It also focused on whether the same results at one year could be achieved by mobilization alone, or if remission required both immune ablation and graft reinfusion. Therefore, patients underwent mobilization and were then randomised either for immediate transplantation (n=23) or for one-year delayed transplantation (n=22).[127] Regarding safety, there was a heavy burden of serious adverse events associated with both the dose of cyclophosphamide used at mobilization and transplantation; one patient died.[127] The most frequent serious adverse events were infections, although there was a decrease in their incidence during the course of the trial.[159] A report from our group shows that adequate supportive care can reduce adverse events, highlighting the importance of conducting HSCT in Crohn's disease at centres with considerable experience.[131]

The ASTIC primary endpoint was sustained disease remission at one year, defined as a complex variable comprised of 3 components: (1) CDAI less than 150 for at least the last 3 months; (2) no active treatment during the last 3 months; and (3) no mucosal erosion or ulceration anywhere in the gastrointestinal tract as determined by a blinded adjudication committee. Despite not achieving this demanding primary endpoint, significant benefits were seen in patients undergoing HSCT compared to mobilization alone. For instance, 61% of HSCT patients achieved clinical remission without treatment for 3 months compared to 23% in the control group (mobilization only, p.value < 0.01). Moreover, 35% of HSCT patients presented no objective evidence of active disease on endoscopy and radiology, compared to 23% in the control group (p. value = 0.053).[127]

As previously mentioned, patients randomized to the control arm in ASTIC were eligible to undergo the HSCT protocol after the primary endpoint had been assessed, and underwent the same schedule of assessments over the following year. A manuscript reporting the combined cohort outcome has recently been published.[159] Compared to baseline, there were highly significant improvements in clinical disease activity, quality of life and endoscopic disease activity at one year after HSCT.[159] Remarkably, 50% achieved mucosal healing, defined as no mucosal erosion or ulceration in all segments examined; and 43% of patients reached steroid-free clinical remission, defined as CDAI < 150.

A report on long-term clinical and endoscopic outcome after HSCT in Crohn's disease is based on a 29-patient cohort studied by in our group.[130] In this cohort, the largest to date from a single center, drug-free clinical and endoscopic remission (CDAI < 150 and global SES-CD < 7 with no segment score ≥ 4, which excludes presence of large ulcers) was seen in 61%, 52%, 47% and 15% at one, 2, 3, 4 and 5 years respectively. Figure 13 shows the efficacy results based on CDAI at all time-points assessed.

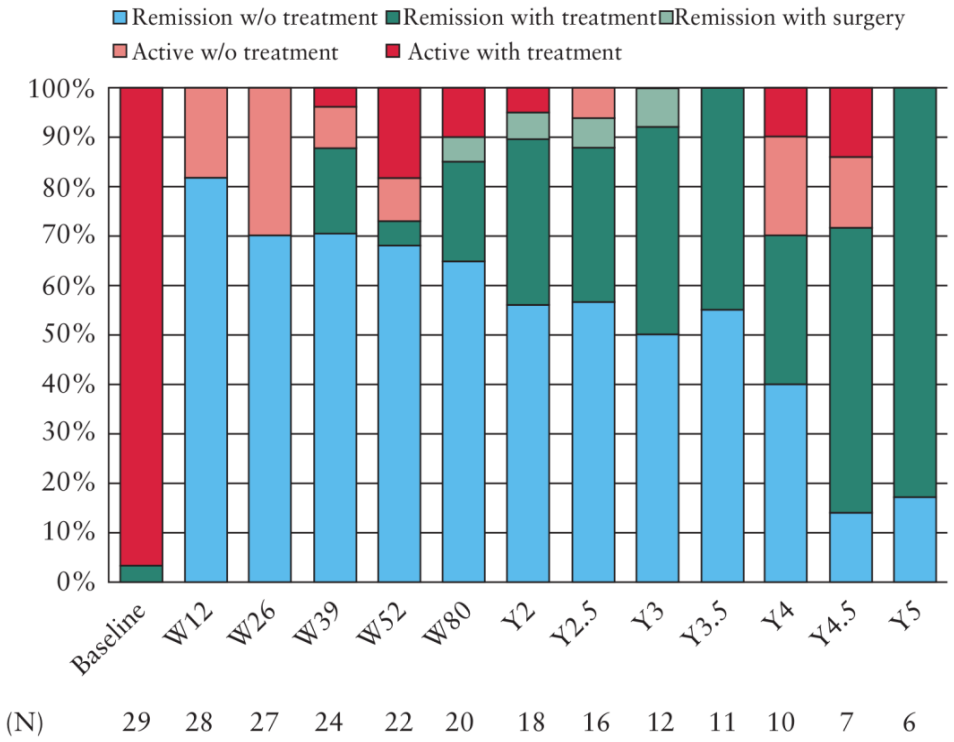
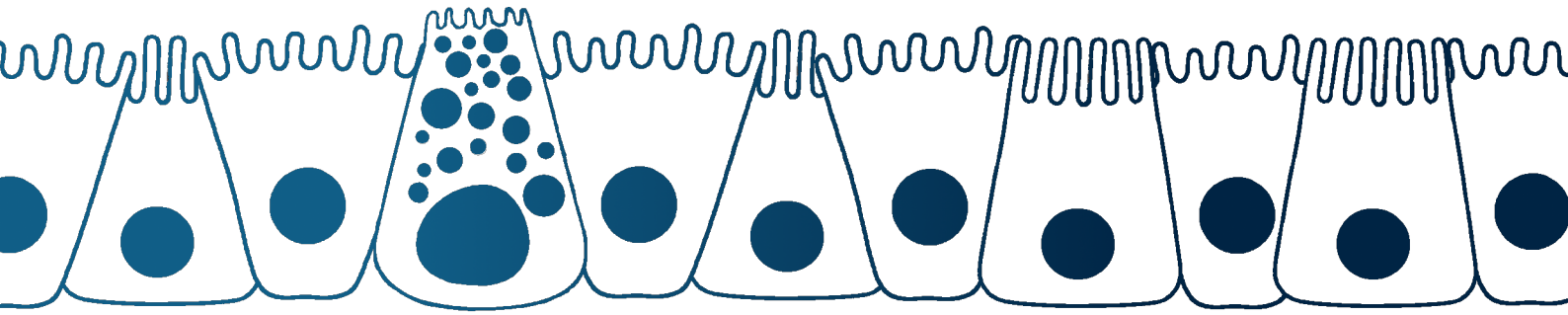


Figure 13. Proportion of patients and disease status based on clinical remission as a CDAI < 150, during 5 years of follow-up. Figure from Lopez-Garcia *et al.*[130]

A recent publication reported the outcome from 82 patients undergoing autologous HSCT for Crohn's disease outside the ASTIC trial.[139] Forty-three percent of patients achieved clinical remission at one year, and 73% of these needed treatment after a median of one year. Fifty-seven percent of treated patients achieved remission or significant symptomatic improvement at their most recent follow-up visit, exhibiting a response to therapies to which they had previously been non-responsive or had lost response.[139]

Hypothesis and Objectives



Hypothesis:

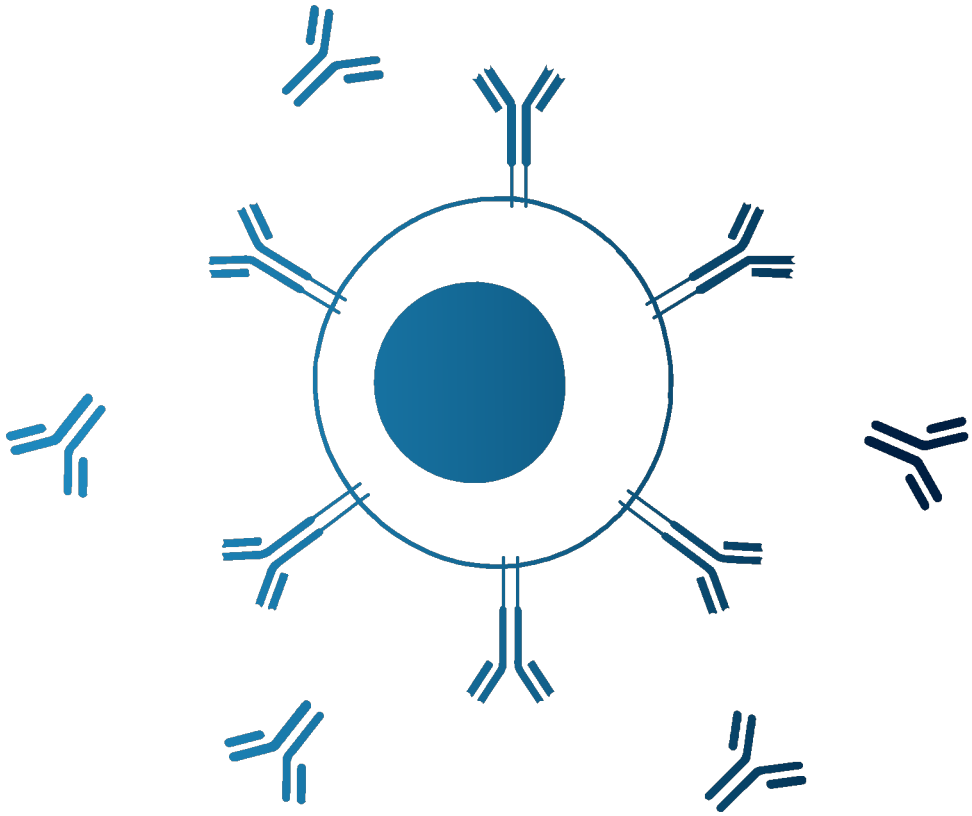
Hematopoietic stem cell transplantation has proven to be successful in inducing drug-free remission in a subset of severe, highly refractory Crohn's disease patients making this approach by far one of the most promising currently available.[127, 130, 138, 139, 154, 155, 156, 157, 158, 159] Despite the benefits this stem-cell therapy can afford, it is associated with a 2-10% mortality rate[6] limiting its applicability to larger cohorts of patients. The mechanisms leading to disease remission after HSCT in Crohn's disease patients are largely unknown, although experience from other autoimmune diseases suggests that "resetting" of the immune response may be involved.

We hypothesize that autologous HSCT exerts a beneficial effect and halts progression of Crohn's disease by eliminating disease-related lymphocytes during the mobilization and conditioning stages of HSCT therapy. In addition, we propose that differential changes in peripheral or intestinal lymphocyte populations may explain HSCT efficacy in Crohn's disease. Finally, we believe that the identification of the mechanism by which autologous HSCT re-establishes intestinal homeostasis in remitting patients will pave the way for the development of targeted and safer cell-based therapies, as well as potentially drug-based therapies.

Objectives:

- Monitor the immune reconstitution of Crohn's disease patients undergoing HSCT in peripheral blood and intestinal biopsies.
- Compare the immune reconstitution and determine the factors associated with treatment success and failure in HSCT.
- Evaluate the TCR and BCR repertoire constitution of patients with Crohn's disease and compare them with healthy non-IBD controls.
- Monitor the eradication and re-appearance of TCR and BCR repertoires in patients undergoing HSCT.

Materials and Methods



Patient eligibility

Autologous HSCT was considered for patients with Crohn's disease who fulfilled the following inclusion criteria:

- active disease at assessment with endoscopic and/or radiological evidence of severe disease activity as defined by the presence of ulcers
- failure or intolerance to all approved medications, including corticosteroids, azathioprine, methotrexate and anti-TNF agents
- unsuitability for surgery due to location and/or extent of the disease or when the surgical option was not accepted by the patient
- impaired quality of life
- signed written informed consent

Those patients with severe comorbidities, symptoms unrelated to Crohn's disease inflammatory activity (i.e., stenosis or short-bowel syndrome-related diarrhea), poor compliance or pregnancy were excluded. A total of 18 patients were recruited between March 2010 and September 2015. Patient characteristics at inclusion are shown in Table 2.

Ethical issues

The use of autologous HSCT for the treatment of Crohn's disease was approved by the regional transplantation organization, Organització Catalana de Trasplantaments (OCATT) on July 25, 2007 and by the local ethics committee on July 22, 2007. Subsequent approval for study continuation was obtained on February 23, 2012. All patients provided written informed consent following extensive counselling.

	All patients	Remitters	Non-remitters
n	18	9	9
Gender (Male/Female)	5/13	1/8	4/5
Age (years) [A]	29.28 ± 1.76	28.89 ± 1.96	29.67 ± 3.05
Age at diagnosis [B]			
A1 (<16years)	6 (33)	3 (33)	3 (33)
A2 (17-40 years)	12 (67)	6 (67)	6 (67)
A3 (>40 years)	0 (0)	0 (0)	0 (0)
Disease behaviour [B]			
Inflammatory	13 (72)	7 (78)	6 (67)
Stenosing	1 (6)	1 (11)	0 (0)
Penetrating	4 (22)	1 (11)	3 (33)
Disease location [B]			
L1 (ileal)	0 (0)	0 (0)	0 (0)
L2 (colonic)	4 (22)	2 (22)	2 (22)
L3 (ileocolonic)	9 (50)	4 (45)	5 (56)
L1+L4 (ileal + upper disease)	1 (6)	1 (11)	0 (0)
L3+L4 (ileocolonic + upper disease)	4 (22)	2(22)	2(22)
Disease duration (years) [A]	10.33 ± 1.26	9.11 ± 1.66	11.56 ± 1.90
CDAI [A]	268.38 ± 25.13	258.30 ± 23.58	278.46 ± 45.85
SES-CD [A]	22.73 ± 1.90	21.14 ± 2.32	24.13 ± 3.04
Mutated NOD_2 (Y/N)	2/16	1/8	1/8

Table 2. HSCT cohort: patients' characteristics at inclusion. [A] Mean ± SEM; [B] n (%)

Patient population and follow-up

After discharge, patients were closely followed-up. Crohn's Disease Activity Index (CDAI) and laboratory markers were assessed weekly during the first 30 days, and every 6 weeks thereafter. Colonoscopy and/or magnetic resonance were performed at baseline and at weeks 26 and 52 after transplant. Simple Endoscopic Score for Crohn's Disease (SES-CD) index was used at baseline and during follow-up to assess endoscopic activity. Mucosal healing was defined as SES-CD <7. Magnetic Resonance Index of Activity was used at baseline and during follow-up in those patients in whom lesions could not be assessed by ileocolonoscopy; mucosal healing was defined as segmental index < 7 in all ileocolonic segments. Data is shown in Table 3.

Patient	Remitter	Week 0		Week 26		Week 52	
		CDAI	SES-CD	CDAI	SES-CD	CDAI	SES-CD
P1	YES	332	26	137	-	88.22	2
P3	YES	236	19	180.29	6	54	0
P4	YES	262.1	34	61.5	4	24.74	0
P5	YES	336.64	19	101	6	1.5	0
P9	YES	278.66	20	70.76	4	36	0
P10	YES	162.89	-	94	-	164.78	-
P11	YES	149.37	-	40.9	-	153.1	-
P12	YES	335	12	14	0	10	0
P13	YES	232	18	153.98	4	32.84	0
P2	NO	419	36	72.76	-	174.45	24
P6	NO	267.7	16	232	9	264.8	16
P7	NO	450	22	390	21	382	21
P8	NO	204.98	32	321	29	77	-
P14	NO	324.6	21	31.9	14	278.92	32
P15	NO	123.4	34	122.88	32	37.2	27
P16	NO	448.31	22	290	11	417	12
P17	NO	133.8	10	98.4	7	87.6	7
P18	NO	134.35	-	14.9	-	10.22	-

Table 3. HSCT cohort: clinical and endoscopic disease activity at baseline and during follow-up.

Sample collection

Blood samples were collected at basal time (pre-mobilization) and every 13 weeks after transplant for up to 1 year of follow-up. Blood was collected into PAXgene tubes and frozen at -20ºC (PreAnalytiX (Qiagen, Spain)). A second blood sample was collected to obtain serum for antibody, cytokine and chemokine determinations. A third sample collected in an EDTA-containing tube to isolate whole blood DNA. An additional 40 ml of blood was used to isolate peripheral mononuclear blood cells.

Colonic and ileal biopsies were collected at the described time points from the involved areas of the intestine of Crohn's disease patients and from the sigmoid colon or rectum of patients without inflammatory bowel disease (non-IBD) controls. Biopsies were taken at routine colonoscopies, placed in RNAlater RNA Stabilization Reagent (Qiagen) and stored at -80°C until RNA isolation. Biopsies were taken whenever possible from the involved mucosa (colonic and/or ileal) of patients undergoing HSCT at different time points (Table 4)

Patient	Week 0		Week 26		Week 52	
	Colonic	Ileal	Colonic	Ileal	Colonic	Ileal
P1					X	X
P2	X	X	X		X	
P3	X		X		X	
P4	X		X		X	
P5	X		X		X	
P6	X		X		X	
P7	X		X		X	
P8	X					
P9	X		X	X	X	X
P12	X		X		X	
P13	X		X	X	X	X
P14	X		X		X	
P15	X		X		X	
P16	X		X		X	
P17				X		X

Table 4. HSCT cohort: biopsy samples collected by patient and time point.

Isolation of peripheral blood mononuclear cells

As mentioned above, 40 ml of blood was used to isolate peripheral mononuclear blood cells (PBMCs) in patients at baseline and at weeks 13, 26, and 52 after HSCT. Whole blood was diluted with 40 ml of Phosphate Buffer Saline (PBS, Gibco, Life technologies). This suspension was carefully deposited on 10 ml of the Ficoll-Hypaque (1.077±0.001g/mL, Sigma) solution in a conical tube. After centrifugation at 690*g* for 20 min without stopping at 20°C, the layer of PBMCs (Figure 14) was harvested using a sterile Pasteur plastic pipette and transferred to a sterile tube. PBMCs were washed twice with PBS and then counted.

Cell viability was assessed using Tripan blue Stain 0.4% (Lonza). Afterwards, cells were suspended at $1x10^6$ cells/ml in X-vivo media (Lonza, X-Vivo 15 with L-glutamine, gentamicin and phenol red) supplemented with 2% of inactivated human serum (Sigma, Human serum type AB male). PBMCs were cryopreserved in 10% dimethyl sulfoxide (DMSO) until later use for cell population analysis.

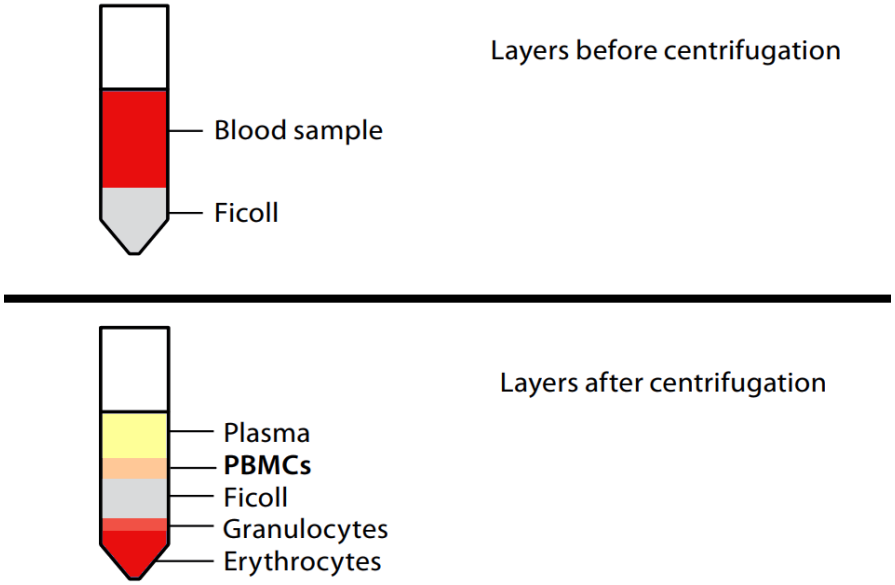


Figure 14. Peripheral blood mononuclear cells isolation. Density gradient formed before and after centrifugation. PBMCs: peripheral mononuclear blood cells.

Flow Cytometry

Flow cytometry was performed to characterize and compare the PBMCs populations' pre-HSCT and at weeks 13, 26 and 52 after HSCT. PBMCs were thawed with X-vivo 15 + 2% Human serum. Cells were twice washed with staining buffer (PBS + 2% fetal bovine serum + 0.01% NaN3) and distributed under different staining conditions (200.000 cells/condition). Viability was assessed using a LIVE/DEAD Fixable Green Dead Cell Stain Kit (Thermo Fisher Scientific) combined with specific antibodies that defined different cell populations (Table 5 and 6).

Cells were washed 3 times with staining buffer after 30 min of incubation on ice and in the dark. Flow cytometry was performed using a FACSCanto™ II, and analyzed with Flowjo v.10™ software. The total number of cells (cells/µl) was

calculated using the percentage of each population obtained by flow cytometry and the absolute lymphocyte count obtained in the clinical blood test run on the same day of the experiment.

Antibody	Fluorochrome	Brand	Reference number
LIVE/DEAD™	FITC	Thermo Fisher	R37601
CD3	V450	BD	560365
CD4	PerCP Cy5,5	BD	552838
CD8	V500	BD	560774
CD19	PerCP Cy 5,5	BD	345778
CD27	PE Cy7	BD	560609
CD38	APC	BD	555462
CD45RA	PE Cy7	BD	561216
CD45RO	APC	BD	559865
CCR7	PE	MILTENY	130-093-621
CD19	APC	MILTENY	130-091-248

Table 5. Fluorescent antibodies used for different cell population analyses.

Fluorochromes	T cell	B cell
FITC	LIVE/DEAD™	LIVE/DEAD™
PE	CCR7	
PerCP-Cy5.5	CD4	CD19
PECy7	CD45RA	CD27
APC	CD45RO	CD38
V450	CD3	
V500	CD8	

Table 6. Antibody combinations used for flow cytometry cell population analyses.

RNA and DNA isolation

Blood RNA isolation was performed with the PAXgene Blood RNA (Qiagen, Spain). DNA was extracted with a Chemagic DNA Blood Kit (PerkinElmer Inc., USA). DNA and RNA were quantified by NanoDrop spectrophotometer (NanoDrop Technologies, USA). The purity and integrity of the total RNA was assessed via a 2100 Bioanalyzer (Agilent, Germany). All RNA samples analyzed had an RNA Integrity Number > 7.

cDNA Synthesis and Real-Time PCR

RNA was transcribed to cDNA using a High-Capacity cDNA Archive RT Kit (Applied Biosystems, USA), and was then used to perform quantitative real-time PCR in triplicate wells with a TaqMan Universal PCR Master Mix (Applied Biosystems) containing the probe of interest and β-actin as an endogenous control gene (TaqMan primers and probes; Applied Biosystems). The following pre-designed TaqMan Assays were used: CD79A, PTK7, IL7R, CCR7, CD40LG, IGHD and CD28. PCRs were performed using an Applied Biosystems 7500 Fast Real-Time PCR detection system. mRNA content (x) was expressed as arbitrary units (AU) and calculated using the formula $x = 2^{-\Delta Ct} \times 10{,}000$; where $\Delta Ct = Ct_{\text{target gene}} - Ct_{\beta\text{actin}}$.

Microarrays

Transcriptomic analysis of whole blood RNA samples was performed at weeks 0, 13, 26 and 52. RNA was hybridized in Affymetrix® chips Human Genome U219. Raw data was analyzed using Bioconductor tools in R (v.3.2.3) employing linear models for microarray data (LIMMA) for differential expression analysis. The CG content-adjusted robust multi-array algorithm was performed on Affymetrix raw-data in order to normalize it, obtaining a $\log_2$ expression value for each probe set. A conservative probe-filtering step was applied to remove low variability probes (coefficient of variation below 25%, n=4661). To correct for multiple testing, the false discovery rate (FDR) was estimated from p. values derived from the moderated t-statistics using the method of Benjamini and Hochberg.[160] A probe was considered differentially expressed when it reached a significance of 5% FDR and showed an absolute fold-change higher than |1.5|.

Pathway analysis was performed for those genes significantly regulated using Ingenuity Pathways Analysis (Ingenuity™ Systems, www.ingenuity.com). Functional Analysis identified the biological functions that were most significant to the data set.

RNA sequencing

Barcoded RNA sequencing libraries were prepared from 100ng total RNA using Illumina's TruSeq stranded mRNA kit according to the manufacturer's instructions. Libraries were subjected to paired-end sequencing (101 base pairs) on a HighSeq-4000 platform (Illumina, San Diego, CA). Quality filtering was performed using cutadapt v.1.7.1; reads were then mapped against the human reference genome using the STAR aligner v.2.5.2a, and a STAR genome directory created by supplying the Ensembl gtf annotation file (release GRCh38.10). Read counts per gene were obtained using RSEM program v.1.2.31 and the Ensembl gtf annotation file. Follow-up analyses were performed using the R (v.3.2.3) statistics tool. Differential expression analysis was performed with the Limma v.3.34.5 and Edger v.3.20.6 packages. The total number of expressed genes was 24,215. To correct for multiple testing, the false discovery rate (FDR) was estimated using the method of Benjamini and Hochberg.[160] A gene was considered differentially expressed when it reached a significance of 5% FDR and showed a fold-change higher than |1.5|.

Deconvolution analysis

CIBERSORT is an analytical tool developed by Newman *et al.*[161] to provide an estimation of the abundances of member cell types in a mixed cell population using gene expression data. We used CIBERSORT v1.01 in order to estimate the proportions of 22 immune cell types in biopsy samples. Analyses were done with 100 permutations, disabled quantile normalization and default statistical parameters. The results were filtered by a maximum p. value of 0.05.

T and B cell receptor repertoire analysis

DNA from whole blood collected from baseline and at 13, 26 and 52 weeks after HSCT from 14 patients in our cohort, as well as one sample from 10 age-matched healthy controls, was used for this part of the analysis. T cell receptor beta (TCRB) and Immunoglobulin Heavy Chain (IGH) sequencing was performed using the ImmunoSEQ platform. Adaptive Biotechnologies® performed the library generation,

deep sequencing, and sequence filtering. TCRβ and IGH sequences were generated with equal amounts of input DNA. Unproductive sequences were filtered out. Sequences were then clustered into unique productive sequences (clonotypes) to determine overall frequencies. Clonotypes were considered high-frequency when their frequency was higher than the 95% confidence interval for 0 occurrence in a binomial test with the number of clonotypes as events for each sample. Clonality was calculated as follows:

$$Clonality = 1 - \frac{\sum_{i=1}^{N} a_i log_2(a_i)}{log_2(N)}$$

Where a_i is the proportional abundance of clonotype i, and N is the total number of clonotypes. Clonality measures how evenly clonotypes are distributed amongst a set of T or B cells. This quantifies how biased a given immune repertoire is towards a particular set of antigens. We used the Morisita-Horn index (M-H; range 0-1) to quantify similarity between samples. Values near 1 represent two identical repertoires. Values near 0 represent dissimilar repertoires.

Serum antibodies determination

Serum antibodies for different vaccines (Table 7) were determined both before and after transplant in 18 patients of the cohort. All tests were performed following the manufacturer's instructions and were analyzed by the Microbiology Laboratory at Hospital Clinic-IDIBAPS, Universitat de Barcelona.

Serum antibodies to ASCA were measured using an enzyme-linked immunosorbent assay (ELISA) at 4 time points (pre-HSCT, 13, 26 and 52 weeks after HSCT) from 18 patients. ELISA plates were coated at 4°C overnight with 4 μg/well of ASCA or with an irrelevant protein for non-specific background subtraction. Plates were blocked with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1.5 hours at room temperature (RT). After washing, serum was added at 1:100 dilution in 0.1% BSA-PBS for 2h at RT. Afterwards, plates were washed and incubated for 1 h with a 1:50,000 dilution of a horse radish peroxidase (HRP)-conjugated goat anti-human gamma chain-specific antibody (Jackson

ImmunoResearch Labs, West Grove, PA, USA). After washing, plates were incubated with tetramethylbenzidine substrate (TMB) (eBioscience, San Diego, CA, USA). Absorbance was read at 620 nm on a microplate reader (Molecular Devices, Sunnyvale, CA, USA). Specific optical density (OD) was calculated for each sample and antigen after subtracting its background OD. Seroreactivity to each antigen was determined as an OD value greater than the mean OD ± standard deviation in a group of healthy controls (n=18).

Vaccine	Test	Brand	Reference
Rubella	IgG ADVIA Centaur	Siemens	08666235
Bordetella pertussis	ELISA IgG/IgA Testkit	Virotech	EC115.00
Mumps virus	ELISA IgG	Vircell	VCM060
Clostridium Tetani	ELISA IgG Testkit	Virotech	EC 124.00
Varicella-Zoster	ELISA IgG	Vircell	VCM089
Measles virus	ELISA IgG	Vircell	VCM054

Table 7. Serum antibody detection kits for the different vaccines tested.

Serum cytokine and chemokine determination

The assay used for cytokine and chemokine determination was the “Human Cytokine/Chemokine Magnetic Bead Panel” from Merck Millipore (ref: HCYTOMAG-60K) following the manufacturer's protocol. Frozen serum samples were completely thawed, mixed by vortexing and centrifuged to remove particulates prior to use in the assay. Premixed beads were sonicated for 30 seconds and vortexed 1 minute before use. This multiplex bead assay ran 25 µl of serum volume for 38 different cytokines available in pre mixed beads. Reconstituted quality controls included in the kit were used in all the plates. A human cytokine standard was prepared with 5 serial dilutions in assay buffer by adding 50 µL of the 10,000 pg/mL reconstituted standard as shown in Figure 15. The 0 pg/mL standard (background) was assay buffer.

In short, samples, standards (2000, 400, 80, 16, and 3.2 pg/mL) and quality controls were incubated with antibody-coated captured beads for 2 hours in an orbital shaker at 750 rpm, at room temperature. Washed beads were further incubated with biotin-labelled detection antibodies for 1 hour, followed by 25 µl of streptavidin-phycoerythrin incubation for 30 minutes. After washing the plate, beads were resuspended in 150 µl of sheath fluid for 5 minutes.

The concentrations of human cytokines in serum samples were measured using Luminex 200™ HTS with xPONENT® software. We analyzed the median fluorescent intensity data using a 5-parameter logistic curve-fitting method in order to calculate the cytokine/chemokine concentrations in samples with MILLIPLEX® Analyst 5.1 (Millipore Corp, St Charles, MO).

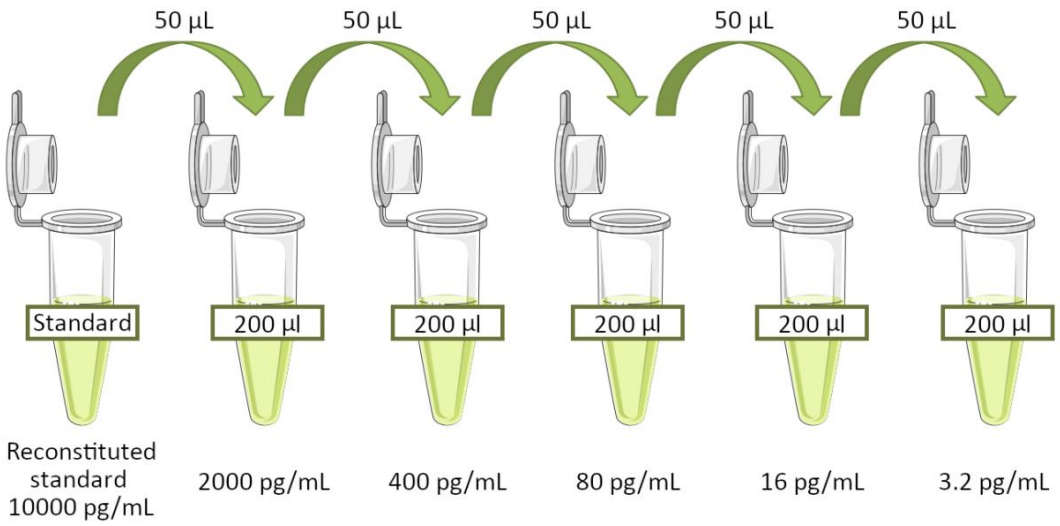
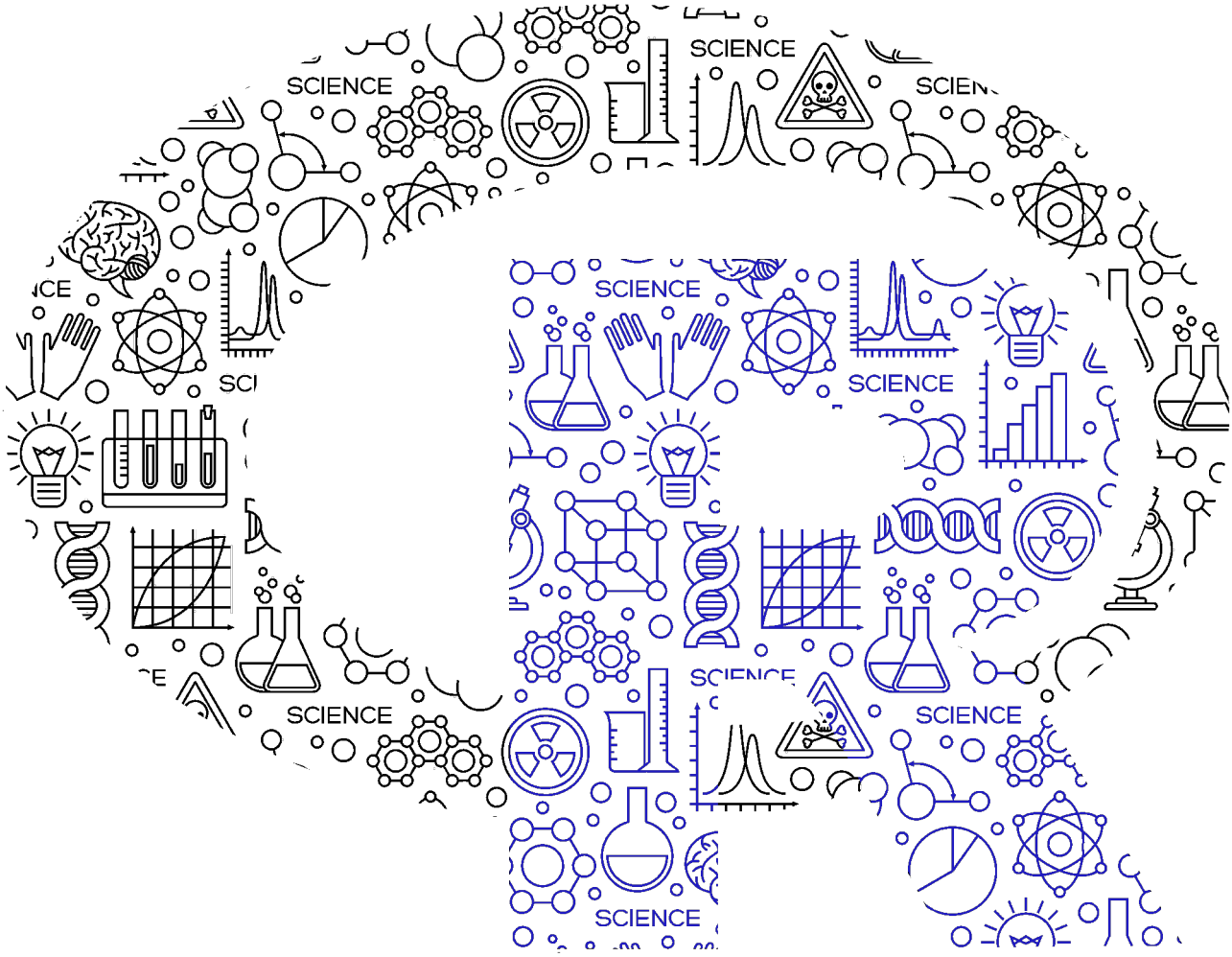


Figure 15. Serial dilution used to prepare the standard curve.

Statistics

For two time-point comparisons, the two-tailed Wilcoxon Rank test for paired samples was used. For two-group comparisons, the two-tailed Mann-Whitney-Wilcoxon test was used. A false discovery rate (FDR) correction for multiple testing was performed when necessary. Nominal or FDR p. values<0.05 were considered significant.

Results



Whole blood transcriptional analysis reflects changes in leukocyte populations following HSCT

We first analyzed the transcriptional signature of peripheral blood by microarray analysis, both before mobilization and immune-ablation (week 0), as well as at different time points after-HSCT (weeks 13, 26 and 52) in 14 Crohn's disease patients from whom blood RNA samples were available (9 remitters). A total of 199 genes were found to be significantly regulated (186 of them were down-regulated) at week 13 compared to week 0. The majority of these genes (95%) returned to baseline levels by week 52 (Figure 16A). Interestingly, when we analyzed the pathways with IPA most of these genes were associated with T cell functions, such as T cell development and the quantity of T lymphocytes (Figure 16B).

A second set of 50 genes (98% of them were up-regulated) showed a delayed modulation, which was significantly regulated at week 26, but not at week 13, compared to week 0 (Figure 16C). The majority of these genes (76%) remained up-regulated at week 52. Pathway analysis of this signature revealed the marked-up regulation of B-cell-related functions, such as the proliferation of B cells and the activation of B lymphocytes (Figure 16D). We compared the transcriptional signatures in the blood of patients categorized by achieving (or not) endoscopic remission one year after HSCT at all time-points studied, but found no significant differences.

The depletion of T-cell-related genes at week 13 closely correlated with changes in the whole T cell populations detected in the peripheral blood of 18 Crohn's disease patients receiving HSCT (Figure 16E). Further analysis revealed that the decrease in total $CD3^+$ lymphocytes at weeks 13 and 26 was primarily due to the sustained depletion of $CD4^+$ cells at those time points (Figure 16E). In agreement with the expansion of the B cell transcriptional signature at week 26, we observed higher B cell numbers in peripheral blood at week 52 after HSCT compared to baseline (Figure 16F). Taken together these results show, as expected, a profound remodeling of the immune cell population after HSCT.

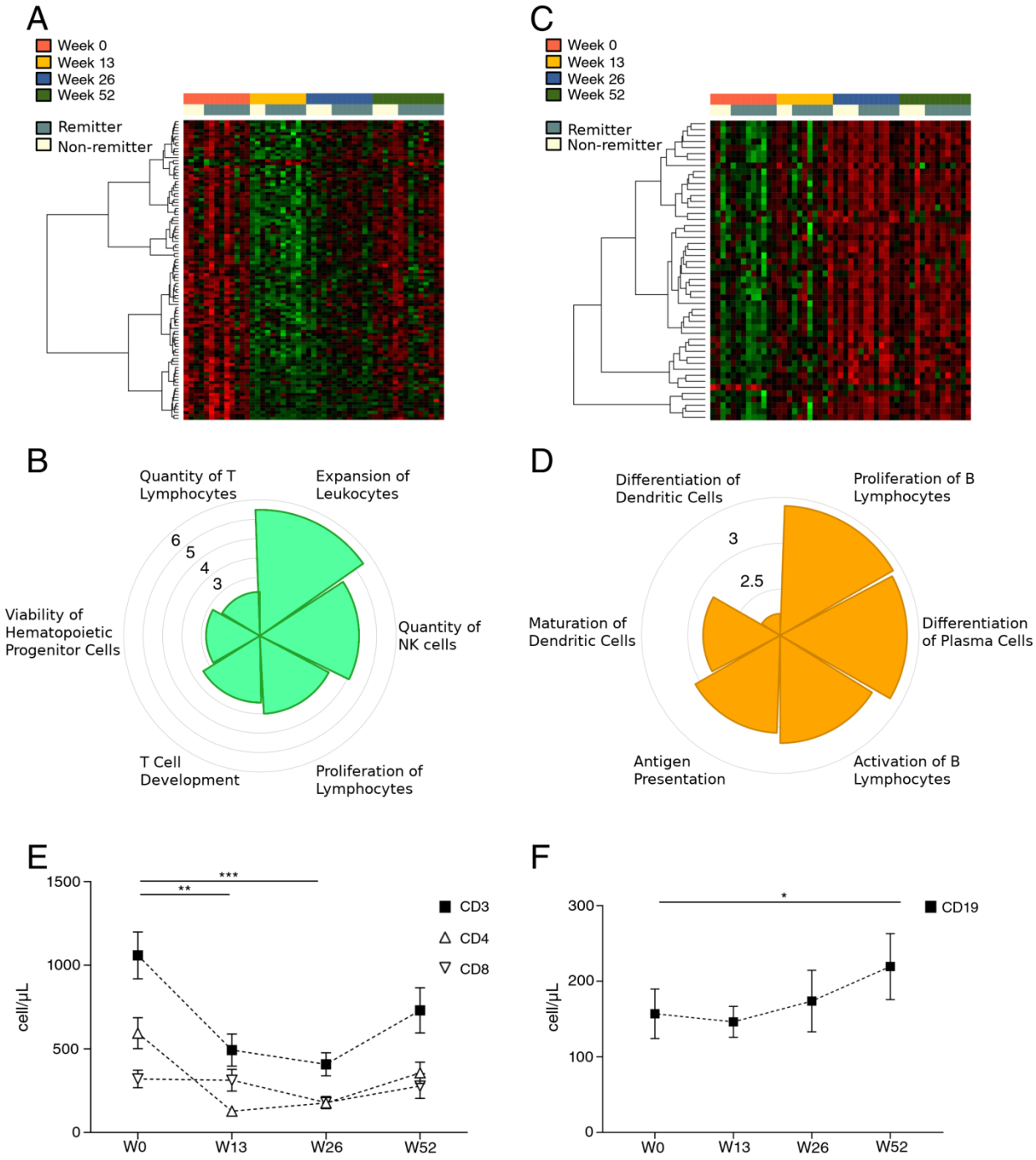


Figure 16. Transcriptional blood signatures following HSCT reveal changes in T and B cells. **A and C**. Heatmap representation of microarray expression of top genes regulated at week 13 (**A**) or week 26 (**C**) compared to week 0. Each row shows one individual probe and each column an experimental sample. High expression levels are shown in red and low expression levels in green. An unsupervised hierarchical cluster method, using a Pearson distance and average linkage method, was applied for each gene classification. **B and D.** Polar graphs showing the top functions identified by Ingenuity Pathways Analysis (IPA) for the genes significantly regulated at week 13 (**B**) and week 26 (**D**). Results are shown graphically as a negative logarithm of the probability score (the most statistically significant pathways have the highest value in the graph). **E.** Absolute numbers of blood $CD3^+$, $CD4^+$ and $CD8^+$ cells/µl (mean ± SEM) at baseline (W0) and after hematopoietic stem cell transplantation (HSCT) in Crohn's disease patients (n=18)). **F.** Absolute numbers of blood $CD19^+$ (mean ± SEM) at baseline (W0) and after HSCT in Crohn's disease patients (n = 18). * p. value < 0.05, ** p. value < 0.01, *** p. value < 0.001; significant by Wilcoxon signed-rank Paired test.

Naïve and effector/memory T and B cells repopulate the peripheral compartment with different dynamics following HSCT

Within the T helper ($CD3^+CD4^+$), the cytotoxic T ($CD3^+CD8^+$) and B ($CD19^+$) cell compartments, we used different cell-surface markers to identify naïve and memory populations by flow cytometry (Figures 17 and 18). At week 13 following transplant, the total number of naïve $CD4^+CD45RA^+$ and $CD8^+CD45RA^+$ cells was significantly reduced compared to week 0 (mean reduction of 96%; p. value = $1x10^{-4}$ for $CD4^+$ cells and 62%; p. value = $6x10^{-3}$ for $CD8^+$ cells). Memory $CD4^+CD45RO^+$ cells were also reduced (mean reduction of 58%; p. value = $2x10^{-4}$), although not to the same extent as the $CD45RA^+$ compartment (Figure 17A). Despite the marked decrease of naïve $CD4^+$ cells, this subset was recovered by 1 year following HSCT. In contrast, $CD4^+CD45RO^+$ cells remained significantly lower at week 52 compared to baseline (p. value = 0.029; Figure 17A), showing that HSCT has a deep and sustained effect on T helper cell populations. On the other hand, $CD8^+CD45RO^+$ cells are increased (mean increase 137%; p. value = $6x10^{-3}$, Figure 17B), and total $CD8^+$ cell numbers are completely recovered at this time point after HSCT (Figure 16E).

Unlike T cells, the proportion of $CD27^-$ naïve B cells increased in peripheral blood following HSCT at the time points studied (mean increase of 49%, 70% and 154% at weeks 13, 26 and 52, respectively; Figure 18). This increase was statistically significant at 1 year post-transplantation (p. value = $3x10^{-2}$; Figure 18), in agreement with the trend shown by transcriptional analysis (Figure 16C and D). In contrast, activated $CD27^+$ B cells were significantly reduced at weeks 13 and 26 (p. value = $7x10^{-3}$ and 0.015, respectively) and had fully recovered by week 52 (Figure 18). This data suggests that the increase we observed in the transcriptional B cell signature in blood most likely resulted from an expansion in the naïve B cell compartment following HSCT.

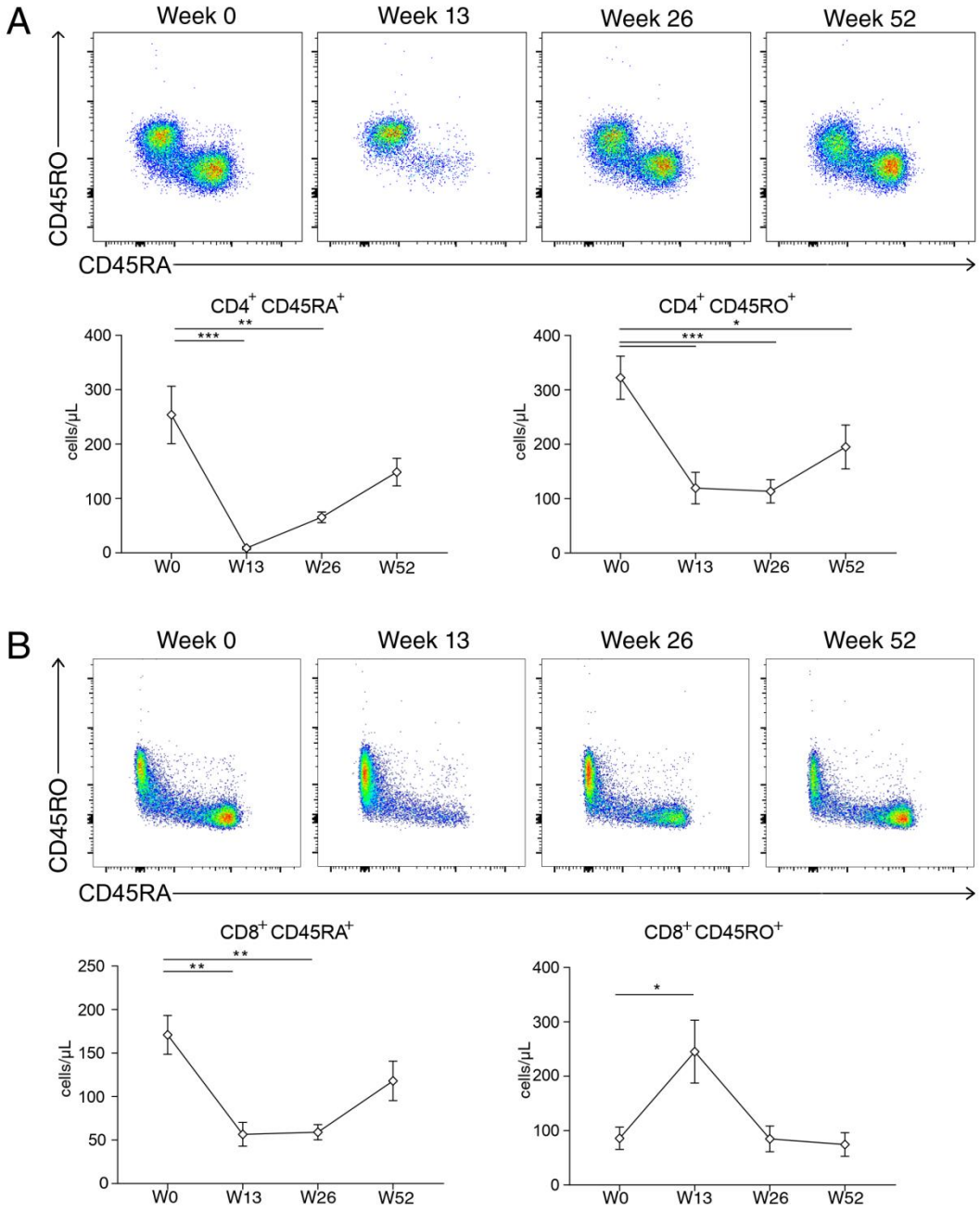


Figure 17. Differential recovery of blood naïve and memory T cells after HSCT in Crohn's disease patients. Dot plots representing naïve ($CD45RA^+$) and memory/activated ($CD45RO^+$) compartments within $CD4^+$ T cells (**A**) and CD8+ T cells (**B**) at baseline (W0) and after HSCT (W13, W26 and W52). Data belongs to one representative patient. Below, the mean ± SEM is represented for all patients included (n=18). * p. value < 0.05, ** p. value < 0.01, *** p. value < 0.001; significant by Wilcoxon signed-rank Paired test.

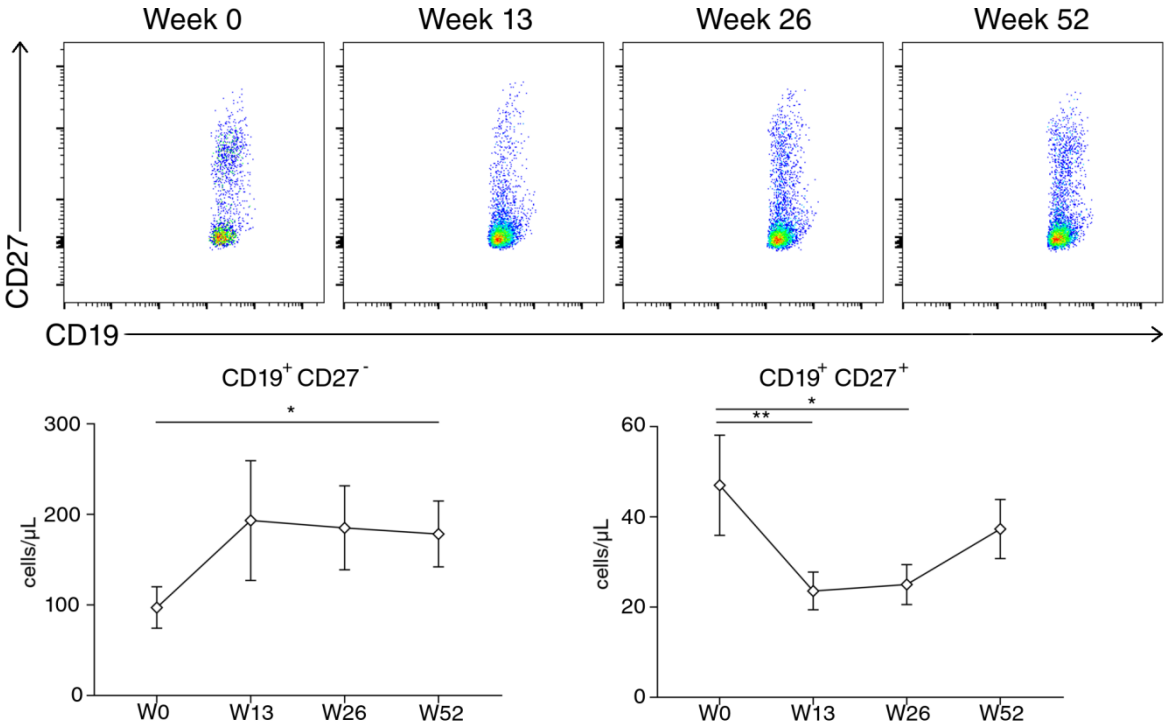


Figure 18. Differential recovery of blood naïve and memory B cells after HSCT in Crohn's disease patients. Dot plots representing naïve ($CD27^-$) and memory ($CD27^+$) $CD19^+$ B-cell subsets at baseline (W0) and after HSCT (W13, W26 and W52). Data belongs to one representative patient. The mean ± SEM for the naïve and memory B cell absolute numbers is represented (n=18). * p. value < 0.05, ** p. value < 0.01, *** p. value < 0.001; significant by Wilcoxon signed-rank Paired test.

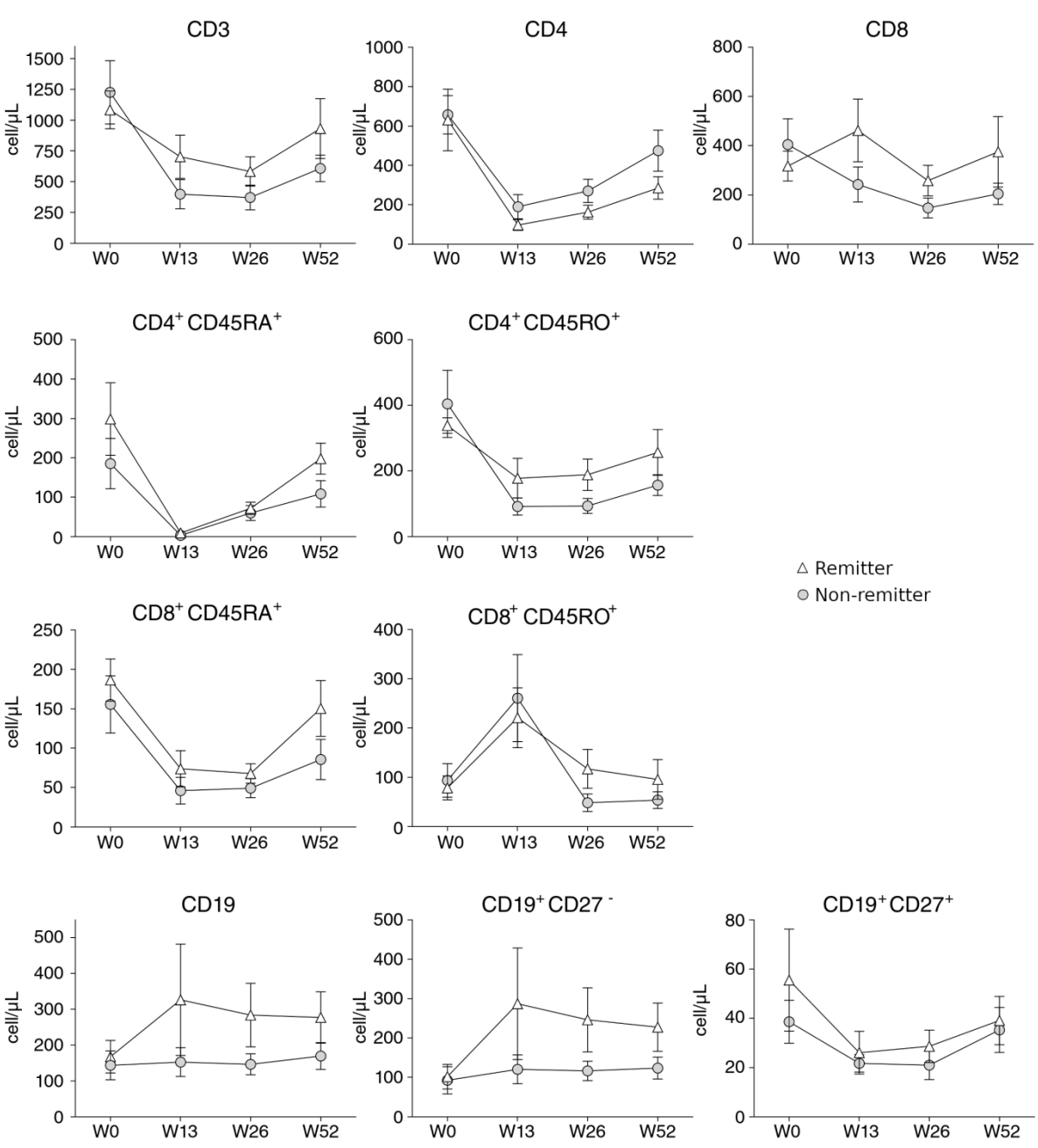


Figure 19. Changes in blood lymphocyte populations in Crohn's disease patients following HSCT categorized by response. Absolute counts of cells determined by flow cytometry in blood (mean ±SEM) at baseline (W0) and after HSCT (W13, W26 and W52). Remitters and non-remitters are shown separately (n=7, per group).

Changes in peripheral blood populations are not related to the control of Crohn's disease activity after HSCT

Fifty percent of our patient cohort achieved endoscopic drug-free remission that was maintained up to 1 year after HSCT (Table 3). As shown in Figure 19, remitters and non-remitters showed comparable changes in cell subsets, including $CD3^+$, $CD4^+$, $CD8^+$ and $CD19^+$ subpopulations, following immune ablation and during reconstitution. In agreement with this observation, the following whole blood transcripts underwent significant changes following HSCT that were comparable in all patients regardless of the protocol's efficacy at all time-points examined: those expressed by naïve T cells (including recent thymic emigrants); i.e., *CCR7* and *PTK7*; *IL7R* and *CD28* genes, both expressed by T cells, and largely by effector memory cells; *CD40LG*, up-regulated by activated T cells; and the B-cell-related genes *CD79A* and *IGHD* (Figure 20). This data suggests that the lack of efficacy of HSCT may be unrelated to the overall measures of immune ablation used in our study.

The majority of cytokine and chemokine concentrations in serum remain unaltered after HSCT

Given the remodeling observed in the lymphocyte populations in our cohort, we measured the concentration of 38 cytokines and chemokines in serum. We analyzed a total of 66 samples from 15 healthy controls and 15 patients at 6 different time points: week 0 (baseline), as well as weeks 6, 13, 26, 39 and 52. First, we compared the serum concentration in patients with active Crohn's Disease (baseline samples) and control samples (n=15). Even though we observed no significant difference, patients tended to have lower concentration of anti-inflammatory IL-10 and higher concentrations of the Th2 cytokine IL-4 (Figure 21).

We analyzed each cytokine and chemokine concentration before and after HSCT with a Kruskal-Wallis test. The majority of them had the same distribution of values in the different groups tested (weeks 0, 6, 13, 26, 39 and 52). Only growth-regulated oncogene (GRO, also known as CXCL1) displayed significant changes in concentration over time (p. value = 0.03, Table 8).

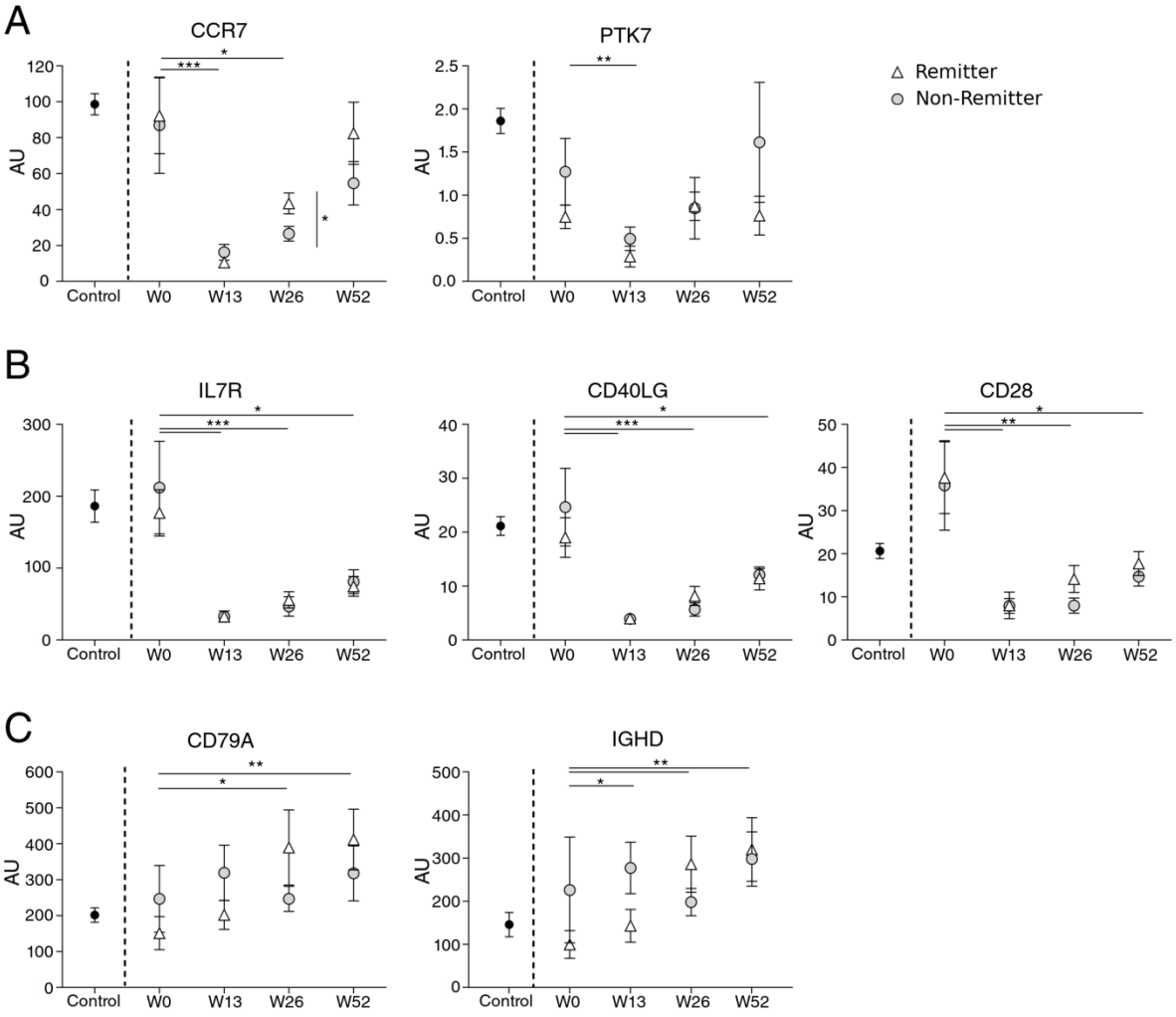


Figure 20. Blood transcriptional analysis of selected T- and B cell-expressed genes following HSCT. Relative mRNA expression (mean ± SEM) of CCR7 and *PTK7* (**A**), *IL7R*, *CD40LG*, and *CD28* (**B**) and *CD79A* and *IGHD* (**C**) in whole blood of 10 healthy non-IBD controls and 18 patients with Crohn's disease undergoing HSCT at baseline (W0) and after HSCT (W13, W26, W52). Gene expression in Crohn's disease patients is shown for remitters and non-remitters separately. Response to HSCT is defined as drug-free endoscopic remission at W52. * p. value < 0.05, ** p. value < 0.01, *** p. value < 0.001; significant by Wilcoxon signed-rank Paired test.

Analyte	W0 (pg/µl)	W6 (pg/µl)	W13 (pg/µl)	W26 (pg/µl)	W39 (pg/µl)	W52 (pg/µl)	p. value
EGF	150.6 ± 88	167.8 ± 107.5	162.4 ± 92.7	178.8 ± 89.5	159.9 ± 99.6	174.4 ± 87.8	0.957
Eotaxin	231 ± 98.2	134 ± 53.1	256 ± 170.4	187 ± 120.5	194.5 ± 173	165 ± 87.4	0.118
FGF-2	87.6 ± 94	21 ± 24.1	22.6 ± 26	20.8 ± 21.1	21.4 ± 27.1	57.5 ± 116.4	0.073
Flt-3L	39.6 ± 95.6	21.2 ± 30.4	50.9 ± 34	20 ± 22.3	38.4 ± 36.4	25.1 ± 34.3	0.37
FRACTALKINE	84.5 ± 129	17.3 ± 35.7	2.2 ± 4.5	99.7 ± 217	10.8 ± 22.7	60.2 ± 120.6	0.366
G-CSF	116.7 ± 257.4	4.4 ± 8.5	17.7 ± 27.7	32.9 ± 59.7	26 ± 46.1	75.6 ± 107.5	0.125
GM-CSF	21.9 ± 37.1	2.1 ± 3.6	3.3 ± 5.8	2.7 ± 5.5	1.9 ± 4.4	7.3 ± 14.7	0.626
GRO	1760 ± 759	1004 ± 285.5	1223 ± 448	1161 ± 447	1272 ± 511	1314 ± 495.9	0.031
IFN-a2	25.4 ± 59.2	0 ± 0	2.9 ± 8.8	3.4 ± 11.3	4.7 ± 13.2	12.6 ± 32	0.55
IFN-γ	42.9 ± 58.4	9.6 ± 14.6	5 ± 6.7	13.9 ± 18.7	14.8 ± 22.2	105.7 ± 337.7	0.823
IL-10	1 ± 2.2	1.5 ± 2.8	0.5 ± 0.9	1.1 ± 3.1	1.1 ± 1.9	1.7 ± 2.8	0.716
IL-12p40	41.8 ± 110.6	41.1 ± 116.2	19.1 ± 57.2	24.3 ± 68.2	28.5 ± 72.2	24.9 ± 57	0.846
IL-12p70	75.9 ± 143.4	0 ± 0	0.1 ± 0.3	37.7 ± 122.5	4.6 ± 11.8	60.3 ± 155.7	0.191
IL-13	18.2 ± 48.4	0 ± 0	0 ± 0	0.5 ± 1.5	2 ± 5.8	3.7 ± 7.8	0.172
IL-15	1.3 ± 2.9	0 ± 0	0.3 ± 0.8	0.1 ± 0.3	0 ± 0	1.3 ± 2.6	0.142
IL-17	32.2 ± 31.7	7.6 ± 12.1	20.8 ± 48.3	24.5 ± 57.3	11.3 ± 20.7	43.8 ± 130.7	0.433
IL-1α	193.2 ± 393	0 ± 0	51.9 ± 155.7	74.2 ± 169.7	39 ± 83.4	53.5 ± 105.8	0.417
IL-1rα	68.6 ± 91.2	24.3 ± 19.6	17.2 ± 17.8	30.6 ± 37.6	34.3 ± 43.3	41.3 ± 44.3	0.205
IL-1β	0.5 ± 1.1	0 ± 0	0 ± 0	0 ± 0	0 ± 0.1	1 ± 2.1	0.288
IL-2	1.7 ± 3.6	0 ± 0	0 ± 0	0 ± 0	0.2 ± 0.7	0.6 ± 1.8	0.323
IL-3	0.2 ± 0.5	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.1 ± 0.4	0.484
IL-4	5.6 ± 13.4	1.3 ± 3.7	0 ± 0	0 ± 0	2.8 ± 8	2.1 ± 3.8	0.222
IL-5	3.7 ± 6.9	0 ± 0	0.1 ± 0.3	0.8 ± 2.3	0.9 ± 1.4	1.2 ± 1.7	0.087
IL-6	24.3 ± 42.6	1.5 ± 4.2	2.8 ± 5.6	2.4 ± 4.7	3.3 ± 6.2	20.8 ± 64.5	0.121
IL-7	25.2 ± 40.8	18.6 ± 51.4	9.8 ± 24.9	11.6 ± 23.1	9.3 ± 18.9	26.6 ± 67.7	0.697
IL-8	58 ± 48.4	28.6 ± 30.2	40.5 ± 65.8	39 ± 62.9	25.2 ± 27.8	39.1 ± 64	0.252
IL-9	1.6 ± 2.8	0 ± 0	0 ± 0	0 ± 0	0.1 ± 0.3	1.5 ± 3.5	0.084
IP-10	322 ± 147.6	445.3 ± 203.5	756 ± 604.1	632.6 ± 456	403.2 ± 227	424.3 ± 226.6	0.151
MCP-1	833 ± 337.3	783 ± 180.8	1090 ± 465	987 ± 424.6	784 ± 451.7	861.4 ± 492.3	0.525
MCP-3	35.3 ± 58.9	1.4 ± 3.9	0 ± 0	7.6 ± 25.2	14.3 ± 40.5	15.9 ± 35.1	0.134
MDC	961.1 ± 436	800.2 ± 346	947.5 ± 318	1082 ± 418	1056 ± 533	1385 ± 973.6	0.513
MIP-1α	11.2 ± 12.4	6.5 ± 8.5	4.6 ± 3.6	8.6 ± 8.1	5.3 ± 6.8	13.5 ± 29.2	0.841
MIP-1β	44.4 ± 32.7	24.6 ± 23.1	21.6 ± 11	27 ± 17.4	16.5 ± 14.5	58 ± 143.1	0.162
sCD40L	10872.3 ± 6182.5	9232.3 ± 3647.6	8600.1 ± 5956.2	10629.3 ± 4841.5	7900.6 ± 3046.3	9146.6 ± 3861.3	0.813
TGF-α	12.2 ± 15.3	2.9 ± 2	2.2 ± 2.1	3.7 ± 5.8	7.6 ± 12.5	6.8 ± 6.5	0.153
TNF-α	18.5 ± 12.3	16.4 ± 11.5	22 ± 12.6	26.3 ± 18.3	20.9 ± 13.2	18.1 ± 11.8	0.788
TNF-β	17.3 ± 43.9	0 ± 0	0 ± 0	2.8 ± 9.2	7.2 ± 20.4	19 ± 41.2	0.167
VEGF	564.4 ± 674	141.1 ± 101.9	175.6 ± 111	188 ± 192.6	178.3 ± 173	520.8 ± 1188	0.718

Table 8. Cytokine and chemokine concentrations in the serum of Crohn's disease patients undergoing HSCT. Mean ± standard deviation. P.value of Kruskal-Wallis test of distribution.

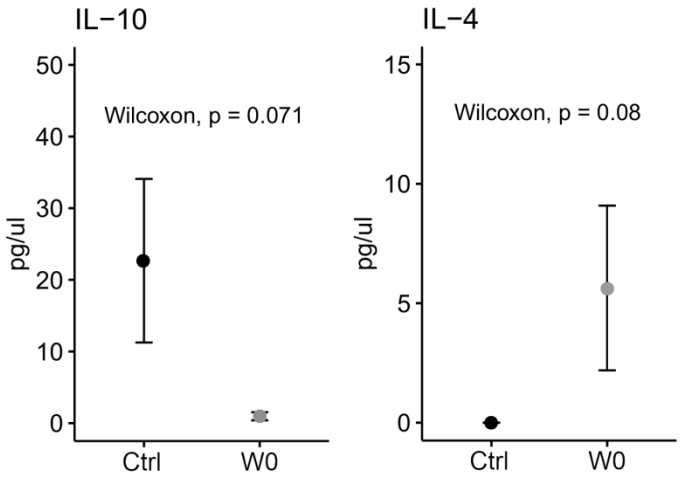


Figure 21. Serum cytokine concentration from controls and Crohn's disease patients in our cohort. Serum IL-10 and IL-4 concentration from 15 controls (Ctrl) and 15 patients with Crohn's disease before transplant (baseline – W0). Mean ± SEM. Wilcoxon rank-sum test was used to compare the groups.

The measurements after treatment compared to baseline revealed that the majority of cytokines (73%, 28/38) remained unaltered over time. We observed a significant decrease of 9 cytokines at the earliest time-point studied after HSCT, including pro-inflammatory cytokines such as IL-6, monocyte-chemotactic protein 3 (MCP3, also known as CCL7), IL-12 active heterodimer (p70), IL-5, eotaxin (also known as CCL11), GRO, fibroblast growth factor 2 (FGF-2), G-CSF and IL-1 receptor agonist (IL-1RA). All of them recovered baseline levels at week 52 (Figure 22). In contrast, pro-inflammatory interferon gamma-induced protein 10 (IP-10, also known as CXCL10) levels were transiently increased at week 13 after HSCT and normalized at week 26 (Figure 22).

Next, we determined whether or not there were different levels of cytokines for remitting and non-remitting patients after HSCT. We compared cytokine concentrations at all time points and observed no significant differences in the recovery of cytokines and chemokine concentration in serum (data not shown). We only observed significant difference in the baseline concentration of IFN-α2, which was lower in non-remitters (Figure 23). Non-remitters tended to have higher concentrations of macrophage-derived chemokine (MDC) compared to remitters. Remitters had significantly lower concentrations of MDC compared to healthy controls (p.value = 0.02, Figure 23). Furthermore, non-remitters tended to have higher concentrations of IL-4 compared to controls (Figure 23).

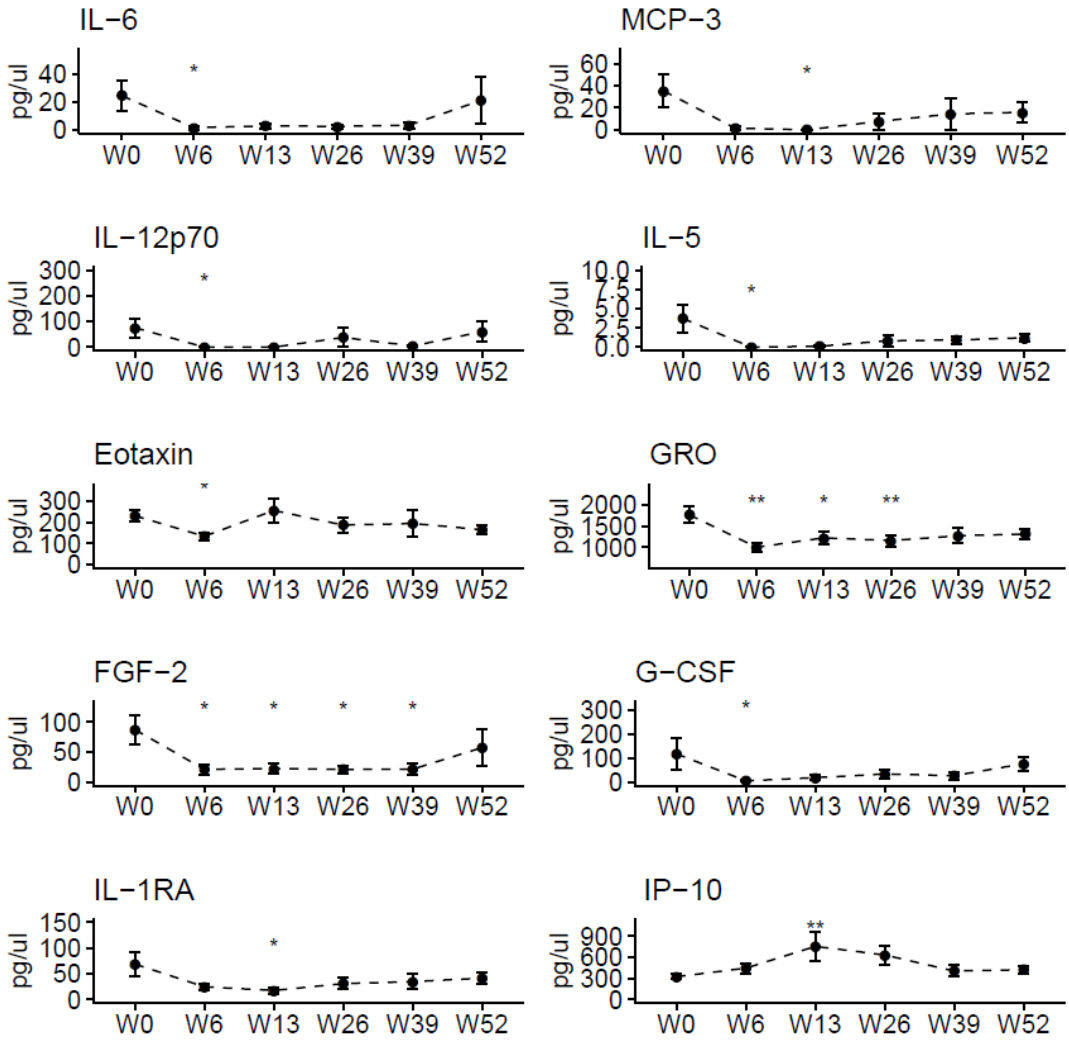


Figure 22. Cytokines significantly changed after HSCT. Serum concentrations of IL-6, monocyte-chemotactic protein 3 (MCP3, also known as CCL7), IL-12 active heterodimer (p70), IL-5, eotaxin (also known as CCL11), growth-regulated oncogene (GRO, also known as CXCL1), fibroblast growth factor 2 (FGF-2), G-CSF, IL-1 receptor agonist (IL-1RA) serum concentrations are reduced after HSCT. On the other hand, interferon gamma-induced protein 10 (IP-10, also known as CXCL10) levels increased at week 13 after HSCT, and baseline serum levels recovered at week 26. * p. value < 0.05, ** p. value < 0.01 significant by Wilcoxon signed-rank test.

In all, serum cytokine and chemokine concentrations could not be used to distinguish between control and inflamed patient samples at baseline or at early time points after HSCT. Only 27% of the cytokines studied had a significant change after treatment. Most of them changed at the first time point studied after transplant and recovered baseline levels one year post-HSCT. Furthermore, IFN-α2 concentration was significantly lower in the baseline serum of remitting patients.

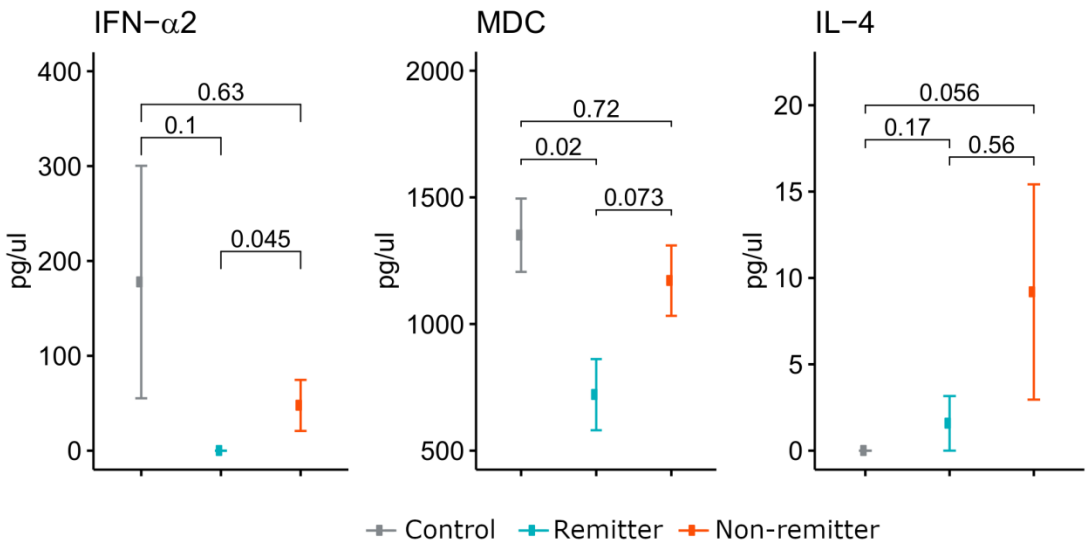


Figure 23. Differential cytokine concentration at baseline based on remission one year after HSCT. Serum concentration of interferon alpha 2 (IFN-α2), macrophage-derived chemokine (MDC) and interleukin 4 (IL-4) in controls (n=15) and Crohn's disease patients at baseline, separated by response (n=7) and no-response (n=8) to HSCT at week 52. P. values were calculated by Wilcoxon signed-rank test.

Transcriptional analysis of the intestine reveals differences between the signatures of remission induced by HSCT and anti-TNF treatments

Biopsies for RNA sequencing (RNAseq) analysis were taken whenever possible from the involved mucosa (colonic and/or ileal) of patients undergoing HSCT at different time points (Table 4). Given that the transcriptomic signatures of colonic and ileal mucosa are markedly different (Figure 24), as well as the fact that the majority of patients had colonic disease (Table 2), we limited our analysis to the colonic signatures for the purpose of this study.

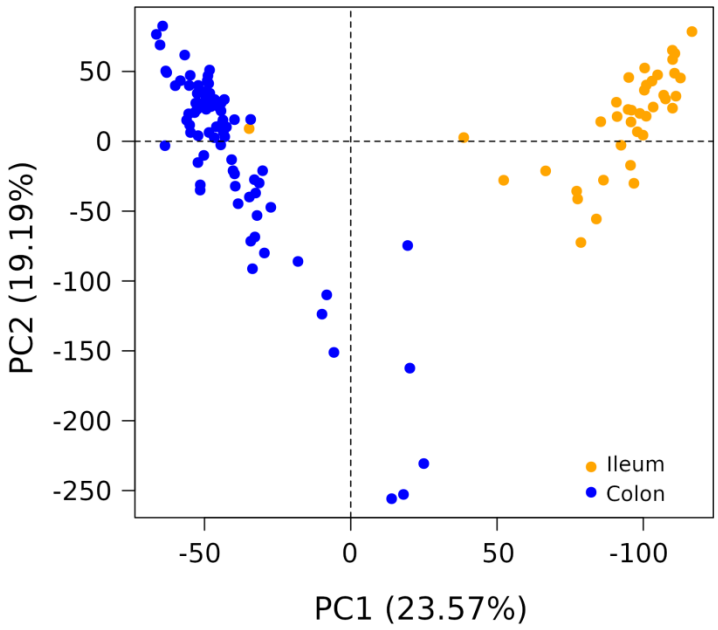


Figure 24. Principal Component Analysis (PCA) of RNA sequencing gene expression profiles derived from intestinal biopsies. Mucosal biopsies were obtained from healthy non-IBD controls and from the involved segments of patients. Ileum samples are shown in yellow (n= 39), and colon samples in blue (n=86). A two-principal component plot is shown with the first component along the X-axis and the second component along the Y-axis.

Differential gene expression analysis was performed between week 0 and different time points (weeks 26 and 52) after-HSCT. In remitters (n=8), a total of 1,504 protein-coding genes were found to be significantly regulated (1,189 of them were down-regulated) at week 26 compared to week 0. At week 52, the number of protein-coding genes significantly regulated from baseline reached 2,099 (1,730 downregulated) (Figure 25A). A common signature containing 1,043 genes was significantly regulated at both time points (Figure 25B shows a heatmap representation of the top 100 regulated common genes).

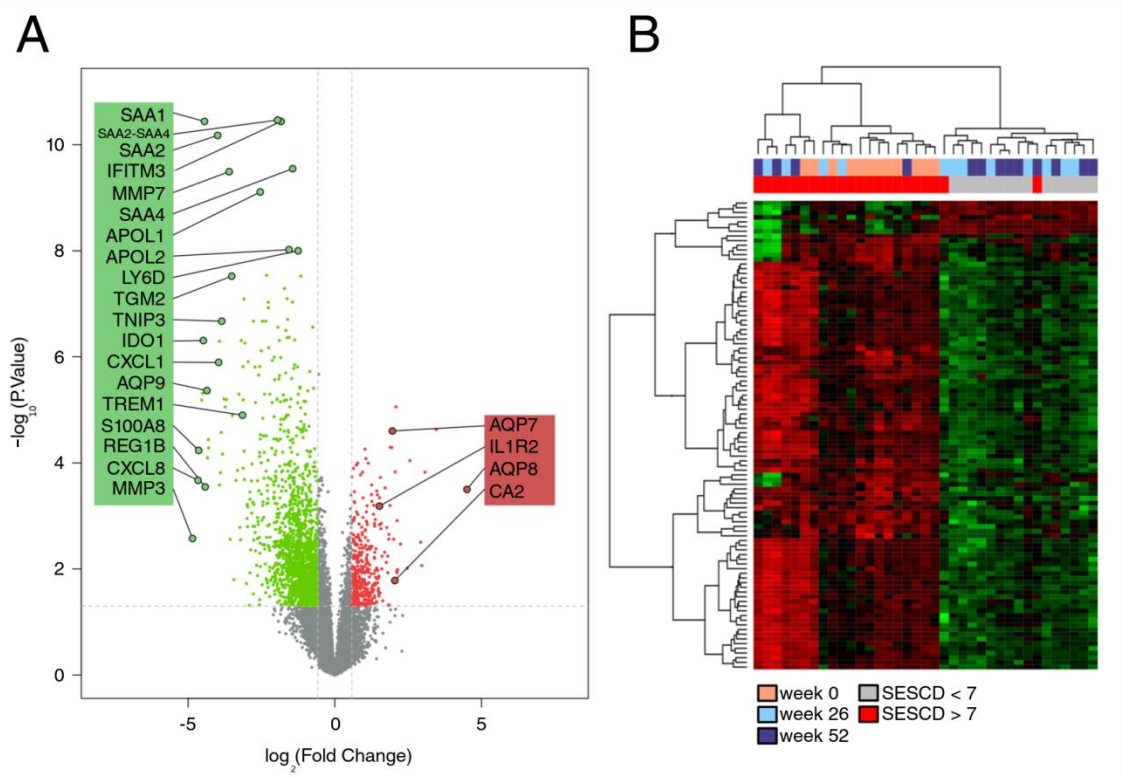


Figure 25. Transcriptional intestinal signatures reveal significant changes after HSCT. **A.** Volcano plot representation of transcriptional changes in biopsies from patients in remission at week 52 compared to week 0. The $\log_2$(fold change) is shown along the x-axis and the -$\log_{10}$(FDR corrected p.value) along the y-axis. Differentially expressed genes that reach significance (considered as FDR corrected $p < 0.05$ and $|FC|>1.5$) are shown in color (downregulated genes at week 52 in green and upregulated genes in red). **B.** Heatmap representation of RNAseq expression of the top 100 genes significantly regulated at week 52 (that were also regulated at week 26) compared to week 0. Each row shows one gene and each column an experimental sample. High expression levels are shown in red and low expression levels in green. An unsupervised hierarchical cluster method, using a Pearson distance and average linkage method, was applied for each gene and sample classification.

Pathway analysis revealed the significant regulation of innate and acquired immune cell activation and recruitment, as well as cytokine production (Figure 26A). Upstream regulator analysis showed the significant regulation of several key mediators in those patients in remission after HSCT (Figure 26B).

The following markers were all predicted to be upstream regulators of those pathways that had been found to be significantly inhibited 1 year post-HSCT: cytokines such as TNF-α, IFNγ, IL-1α, IL-1β, IL-17A, IL-6, transforming growth factor beta (TGFβ), among others; transcriptional factors as nuclear factor kappa beta (NF-κB), STAT1 and STAT3, hypoxia-inducible factor-1 (HIF-1) and interferon regulatory factor 1 (IRF1); or enzymes such as prostaglandin synthase 2 (PTFS2) and transglutaminase 2 (TGM2).

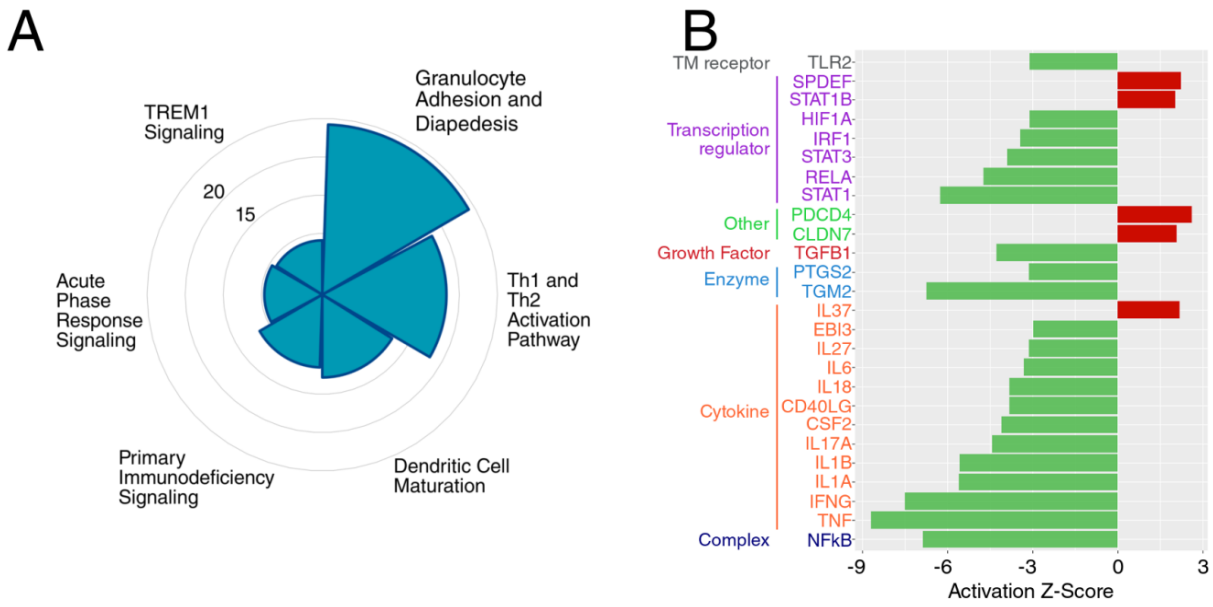


Figure 26. Pathway analysis of the biopsies transcription reveals changes in the immune system. **A**. Polar graph showing the top canonical pathways identified by IPA significantly regulated in remission at week 52 compared to week 0. Results are shown graphically as a negative logarithm of the probability score (the most statistically significant pathways have the highest value in the graph). **B**. Bar plot showing the activation z-score of the top upstream regulators at week 52 in patients in endoscopic remission compared to week 0 found by IPA. The activation z-score infers the activation states of predicted transcriptional regulators. The bases for this inference are the relationships in a molecular network that represent the experimentally observed gene transcription events and their association with the literature-derived regulation direction. If the z-score is negative, the upstream regulator is likely to be inhibited; if positive, activated.

We next compared the signatures of endoscopic remission obtained following HSCT with those induced by anti-TNF treatment. To that end, RNA sequencing analysis of biopsies was performed in a cohort of patients evaluated at baseline and at weeks 14 and 46 following anti-TNF.

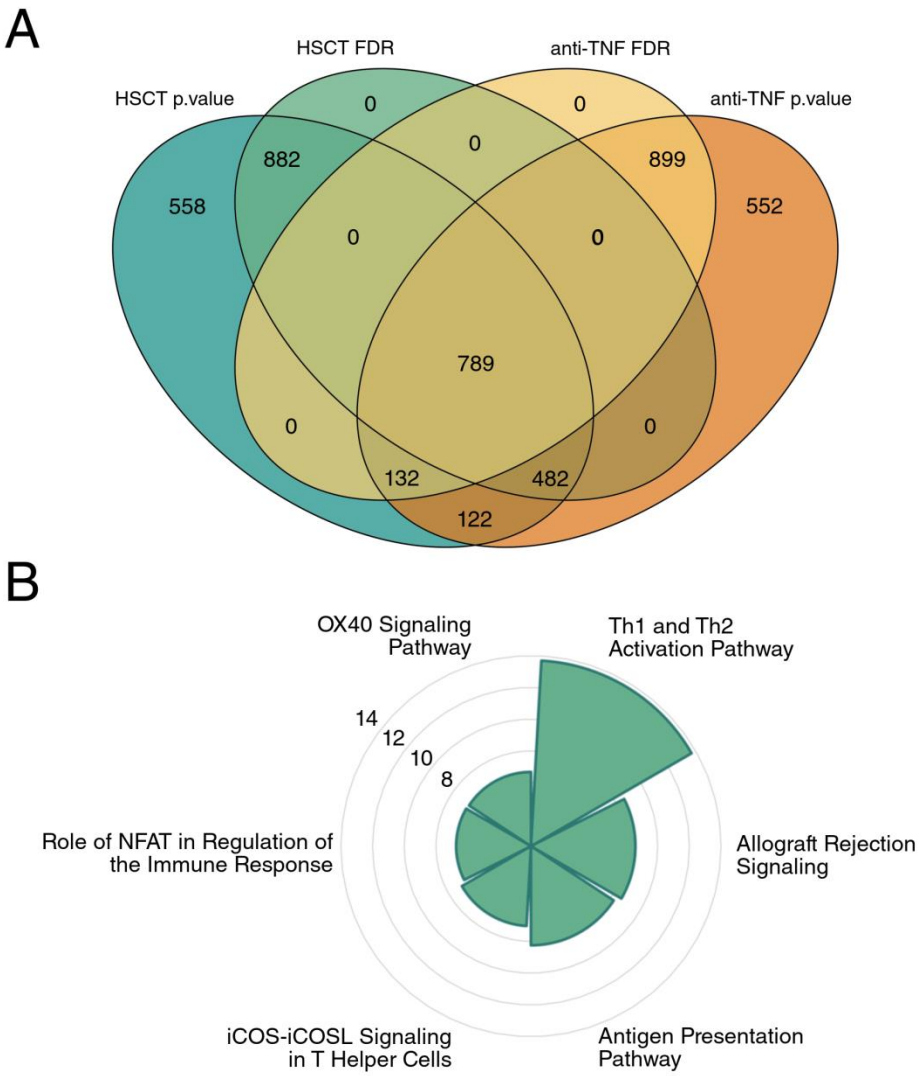


Figure 27. HSCT exclusive remission-induced gene signature is related to the down-regulation of T cell canonical pathways. **A.** Venn diagram showing the number of genes that are differentially expressed in Crohn's disease remitting colonic mucosa at week 52 after HSCT or week 46 after anti-TNF treatment compared to their respective baseline expression values. Genes are classified based on their significance: FDR < 0.05 or p < 0.05. About 40% of the genes (882 transcripts) within the HSCT induced signature were not changed by anti-TNF. **B.** Polar plot representing the top six pathways significantly regulated in the HSCT-induced signature that are not regulated by anti-TNF. The negative logarithm of the probability score (the most statistically significant pathways have the highest value in the graph) for each pathway is represented.

Differential gene expression analysis of endoscopic remission at 46 weeks compared to baseline (pre-treatment) showed the significant regulation of 1820 genes. Forty-three percent of this gene signature (789 genes, Figure 27A, Annex Table 1) was also regulated during remission following HSCT.

Moreover, 58% of the 2,099 genes that changed (FDR<0.05; FC > 1.5) at week 52 due to HSCT were also regulated, though only when uncorrected by multiple comparisons (nominal p. value < 0.05 FC > 1.5) in anti-TNF induced remission (Figure 27A). Nonetheless, we observed a large number of genes (882) whose expression was exclusively regulated in remitters of the HSCT cohort. Among these HSCT-only regulated signatures, we observed the significant down-regulation of T-cell-related transcripts (i.e., *CD3E*, *CD28*, *CD3G*, and *CD4*). Indeed, Ingenuity Pathway Analysis revealed the marked inhibition of canonical pathways involving T-effector functions (Figure 27B, Annex Table 2), suggesting that either the amount of T cells or their degree of activation was severely diminished in HSCT-induced remission.

Comparison of genes regulated at 1 year of follow-up compared to baseline in anti-TNF and HSCT showed a very low number of common genes in the non-remitter population; only 26 out of the over 1,600 regulated (FDR<0.05) after HSCT were also changed by anti-TNF (Figure 28).

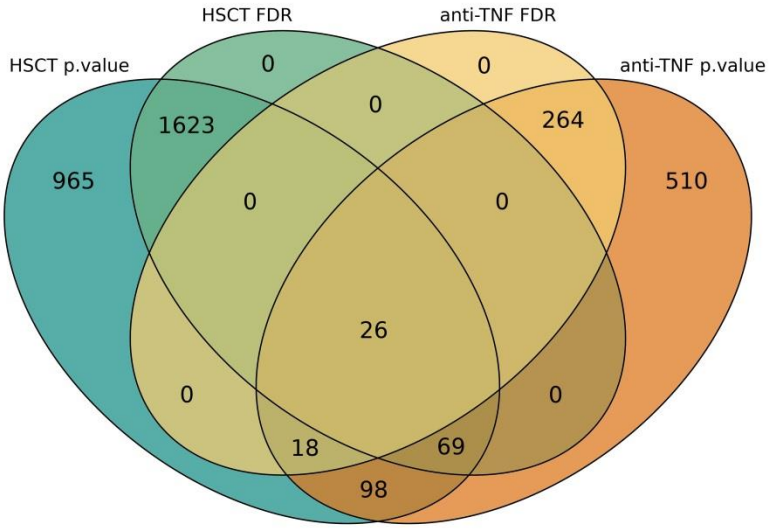


Figure 28. Non-remission gene signature responses to HSCT or anti-TNF share a very low number of genes. Venn diagram showing the number of genes that were differentially expressed in Crohn's disease non-remitting colonic mucosa at week 52 after HSCT or week 46 after anti-TNF treatment compared to their respective baseline expression values. Genes are classified based on their significance: FDR < 0.05 or p. value < 0.05

Immune cell deconvolution analysis reveals unique changes in patients responding to HSCT treatment compared to anti-TNF

In light of the differences in the transcriptomic remission signatures between anti-TNF and HSCT treatments, we decided to analyze the changes in proportions of immune cells in the intestinal mucosa by CIBERSORT deconvolution analysis of the available biopsy RNAseq data. In both cohorts, a subset of samples from non-IBD controls were also sequenced side-by-side for comparison.

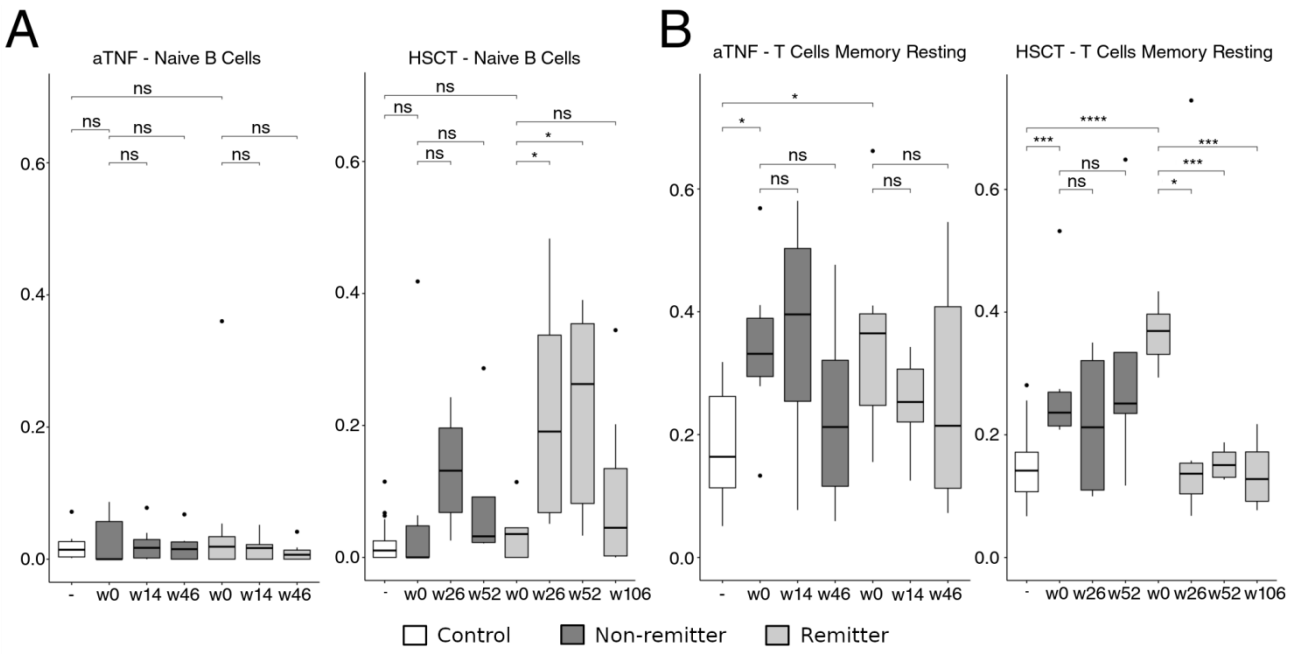


Figure 29. Immune cell deconvolution analysis reveals unique changes in intestinal cellular composition in patients responding to HSCT treatment compared to anti-TNF. Cell deconvolution analysis (CIBERSORT) of biopsy samples from healthy non-IBD controls (n = 19) and Crohn's disease patients before and after treatment with anti-TNF (n = 22, 13 remitters) and HSCT (n = 14, 8 remitters). The figure shows the results for naïve B cells (**A**) and resting memory T cells (**B**). The information on colon biopsy samples from remitters to HSCT at week 106 (2 years following HSCT; n = 7) is also included in this analysis. * p. value < 0.05, ** p. value < 0.01, *** p. value < 0.001, **** p. value < 0.0001; significant by Mann-Whitney-Wilcoxon test.

We observed a significant increase of “naïve B cells” at 6 months and 1 year following HSCT (Figure 29A) that seemed to closely parallel the expansion observed in peripheral blood (Figure 18). In addition, patients responding to HSCT exhibited a significant decrease in the “T cell memory resting” subset (Figure 29B). None of the anti-TNF-treated patients, regardless of response, showed comparable changes in these lymphocyte subsets.

Naïve B cells that were expanded up to 1 year following transplant returned to pre-transplant levels at 2 years (week 106) of follow-up (Figure 29A). In contrast, the decrease in the T cell signature remained constant even up to 2 years after HSCT (Figure 29B). Hence, deconvolution analyses revealed significant changes both in the B and T cell mucosal compartments in patients in remission after HSCT, but not in those responding to anti-TNF.

In contrast, when we looked at innate cells, which are abundant in the inflamed mucosa of patients with active Crohn’s disease, we observed a significant decrease of M1 macrophages and neutrophils. This occurred in both the HSCT and the anti-TNF responding cohorts, correlating with endoscopic remission in both groups of patients (Figure 30). Taken together, these deconvolution results suggest that changes both in the B and T cell mucosal compartments are not alterations common to a generalized control of inflammation, but are rather unique to HSCT therapy.

In order to better characterize the changes in the T cell subsets following HSCT or anti-TNF, we compared the expression of a selection of T cell-related (Figure 31A, B and C) and myeloid-related (Figure 31D) transcripts as measured by RNAseq in both cohorts of patients. While expression of the neutrophil or macrophage markers *CXCL9*, *CXCL10*, *CD16B* was significantly regulated in anti-TNF- and HSCT-responding patients (but not in the non-remitter groups, Figure 31D), the T cell markers *CD3E*, *CD28* and *CD40LG*, as well as the T resident memory (TRM) marker *ITGA1* were significantly regulated only in the HSCT-responding cohort.

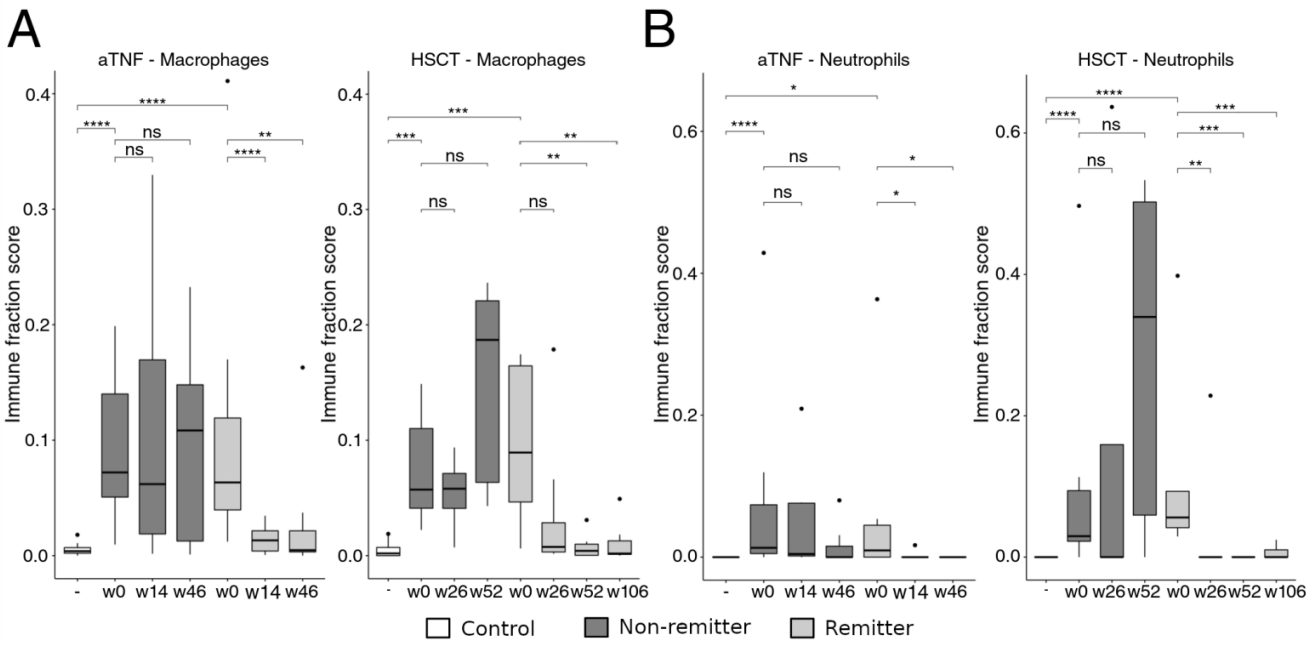


Figure 30. M1 macrophages and neutrophils levels are reduced equally in patients responding to HSCT treatment or anti-TNF. Cell deconvolution analysis (CIBERSORT) of biopsy samples from healthy non-IBD controls (n = 19) and Crohn's disease patients before and after treatment with anti-TNF (n = 22, 13 remitters) or HSCT (n = 14, 8 remitters). The figure shows the results for M1 macrophages (**A**) and neutrophils (**B**). The information on colon biopsy samples from HSCT-remitters at week 106 (2 years following HSCT; n = 7) was also included in this analysis. * p. value < 0.05, ** p. value < 0.01, *** p. value < 0.001, **** p. value < 0.0001; significant by Mann-Whitney-Wilcoxon test.

Two other TRM receptors, *CD69* and *ITGAE*, tended to decrease in the HSCT-remitting group, although this was not statistically significant (Figure 28B). Remarkably, the expression of activated effector T cells such as *S1PR1*, *IL2RA* and *CTLA4* were significantly regulated in all patients who achieved endoscopic response regardless of treatment option (Figure 31C). Overall, our analysis suggests that endoscopic remission in anti-TNF- or HSCT-responding patients is associated with a significant decrease in neutrophil, M1 macrophages and activated T-effector cell markers. In contrast, we propose that HSCT has a profound effect on a subset of memory resting and potentially resident T cells that correlates with response to this therapeutic strategy. HSCT seems to markedly decrease the total T cell content in the mucosa of patients who achieve remission.

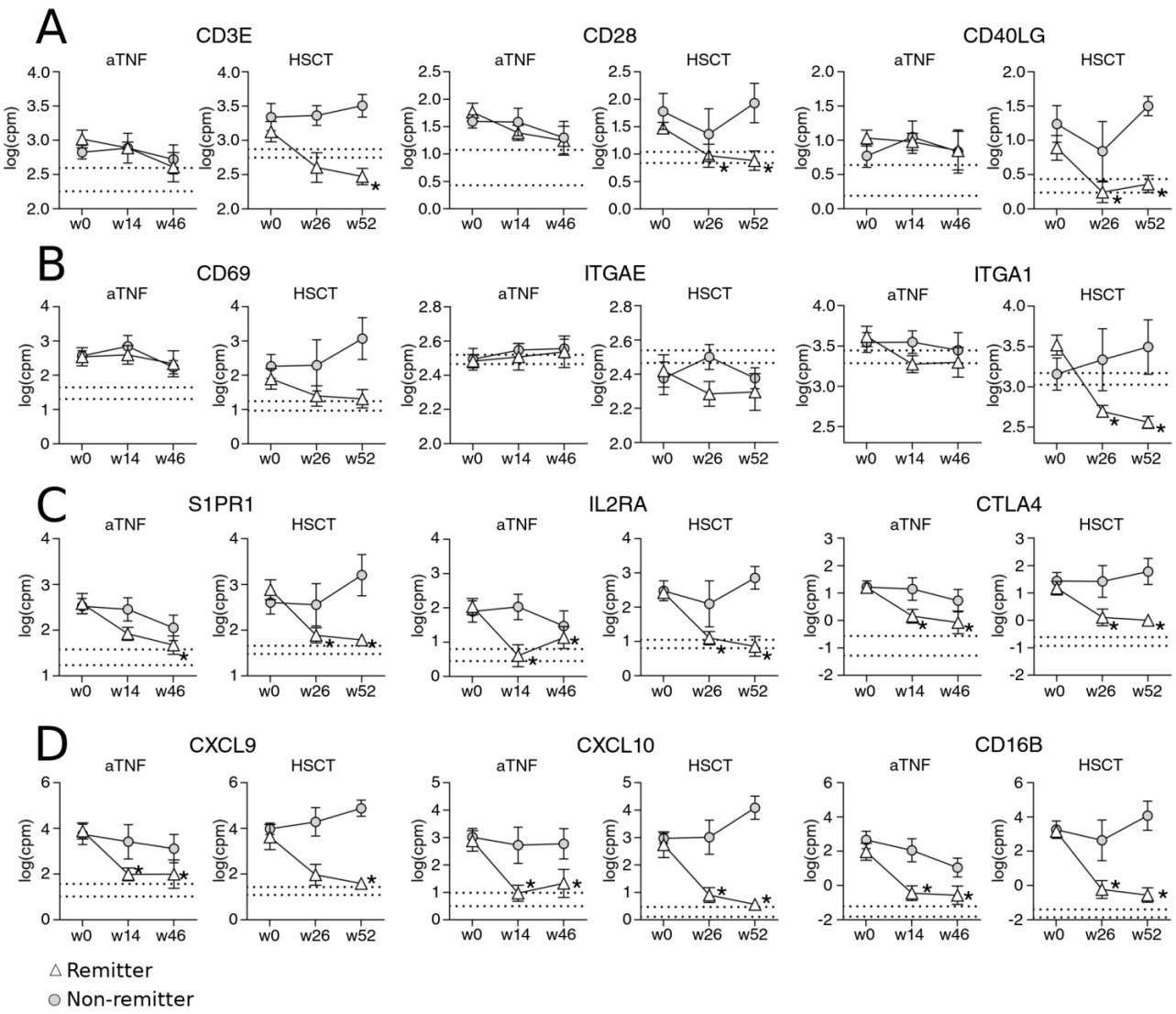


Figure 31. T-cell-related gene expression by RNA-sequencing before and after treatment with anti-TNF or HSCT. Gene expression was determined in intestinal biopsies by RNAseq in Crohn's disease patients treated with anti-TNF therapy or autologous HSCT. The expression values are normalized and represent logarithmic transformed counts for each gene. Dotted lines show the SEM for each gene from the control samples in each cohort. Expression at different time points for endoscopic remitters and non-remitters is shown for the T-cell-related genes *CD3E*, *CD28* and *CD40LG* (**A**); tissue-resident T cells (TRM) *CD69*, *ITGEA* and *ITGA1* (**B**), activated effector T cells *S1PR1*, *IL2RA* and *CTLA4* (**C**), and neutrophils or the macrophage genes *CXCL9*, *CXCL10* and *CD16B* (**D**). Asterisks mark the statistically significant differences in each group for each treatment compared to baseline, by Mann-Whitney-Wilcoxon test * p. value <0.05.

Low TCR diversity at baseline is associated with a lack of response to HSCT

To investigate the effects of HSCT on T cell survival and specific clonal deletion, we obtained the DNA from blood (at baseline and 13, 26 and 52 weeks after HSCT) and mucosal samples (at baseline and 26 and 52 weeks after HSCT) and sequenced the CDR3 β-chain variable regions of the T cell receptor. Using this approach each sequence was assigned to a different T cell clone and each clone's expansion was calculated. With this information, we estimated each patients' TCR diversity using Fisher's unseen species method[162] at different time points.

As expected, total clonotypes (defined as unique *TCRB* nucleotide sequences) were significantly reduced in blood at weeks 13 and 26 compared to baseline and age-matched controls (Figure 32A). The depletion of T cells was also associated with a significant reduction in TCR diversity at weeks 13 and 26 compared to baseline and controls (Figure 32B). The decrement in diversity after HSCT could be due to an expansion of the remaining cells. Patients regained periphery baseline numbers of TCR clonotypes and diversity at week 52.

Neither the number of clonotypes nor TCR diversity in biopsies was significantly lower after treatment (Figure 32). Nonetheless, both measures tended towards a reduction at week 26. This suggests that either T cell deletion was less pronounced in the tissue or that the number of clonotypes recovered at 26 weeks after treatment, the earliest time point studied in this compartment.

Next, we asked if the changes observed over time in the cohort were related to treatment efficacy. Therefore, we analyzed separately patients who achieved endoscopic remission one year after HSCT versus those who did not. This analysis revealed both lower peripheral total clonotypes and TCR diversity at baseline in non-remitting patients (p. value = $4.1x10^{-3}$ and p. value = $2.3x10^{-3}$, respectively; Figure 33). This difference was maintained after HSCT (p.value = 0.01 and 0.01 in clonotypes, and p. value = 0.011 and 0.035 in diversity, at week 26 and 52, respectively).

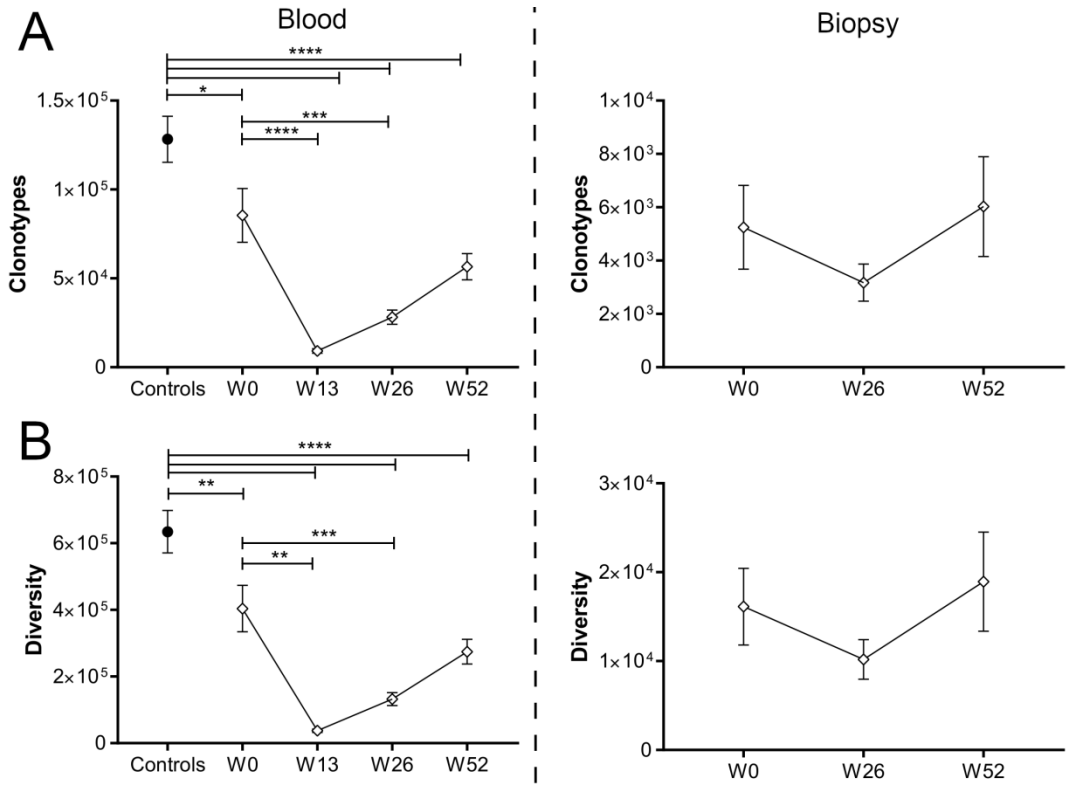


Figure 32. Autologous HSCT induces a more pronounced reduction of TCR diversity in blood compared to intestinal mucosa. **A**. Number of clonotypes, defined as unique sequences, in Crohn's disease patients blood (left, n=14, except W13 n=8) at baseline (W0) and at weeks 13, 26 and 52 after treatment; and biopsy (right, n= 10, except W0 n=9) at baseline and at weeks 26 and 52 after treatment. Whole blood of non-IBD controls (n=10) was included for comparison. **B**. TCR diversity score for controls and patients in blood (left) and biopsy (right). Mean ± SEM are shown. Wilcoxon signed-rank paired test was used to compare patients at different time points and a Mann-Whitney U test was used to compare patient and control samples * p. value < 0.05, ** p. value < 0.01, *** p. value < 0.001, **** p. value <0.0001

Altogether, these results suggest that a highly oligoclonal repertoire at baseline in blood may be a predictor of poor response. Moreover, we show that low repertoire diversity does not improve after HSCT in patients who do not achieve remission after HSCT.

In contrast to blood, in baseline mucosal samples the number of clonotypes or diversity was not significantly different (Figure 33), although there was a trend towards higher numbers in non-remitters in both cases. One year after treatment, these patients had higher number of clonotypes and trended towards higher diversity compared to remitters (p.value = 0.03 and 0.0823, respectively, Figure 33).

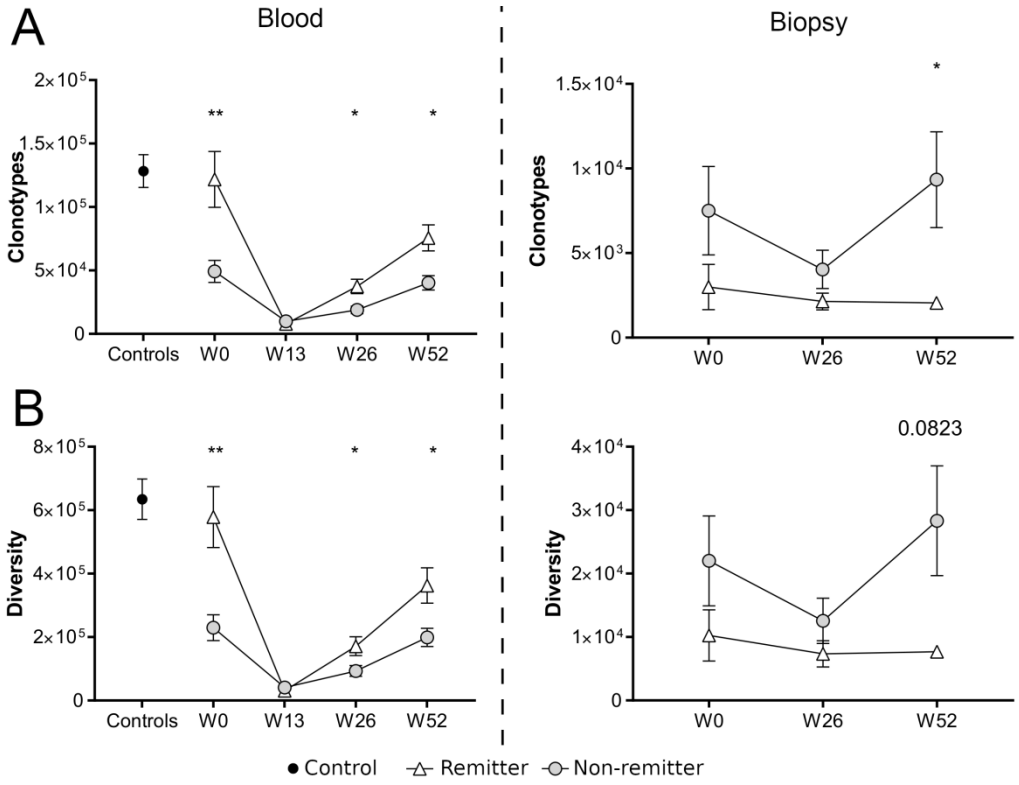


Figure 33. Diversity of the TCR repertoire is significantly lower in no-remitters after HSCT. **A**. Number of clonotypes, defined as unique sequences, in Crohn's disease patients blood (left, n=14, except W13 n=8) at baseline (W0) and at weeks 13, 26 and 52 after treatment; and biopsy (right, n= 10, except W0 n=9) at baseline and at weeks 26 and 52 after treatment. Whole blood of non-IBD controls (n=10) was included for comparison. **B**. TCR diversity score for controls and patients in blood (left) and biopsy (right). Mean ± SEM are shown. Wilcoxon signed-rank paired test was used to compare patients at different time points and a Mann Whitney U test was used to compare patient and control samples * p. value < 0.05, ** p. value < 0.01, *** p. value < 0.001, **** p. value <0.0001

In order to quantify the expansion of the clonotypes present in the samples, we calculated the sample clonality. This index ranges from 0, representing a completely polyclonal sample, to 1, meaning the sample has only one expanded clonotype. In a pool of 10 non-IBD age-matched controls, the clonality index was 0.058±0.039 (mean ± SEM; Figure 34) reflecting the highly polyclonal composition of the T cell pool in the periphery of healthy donors.

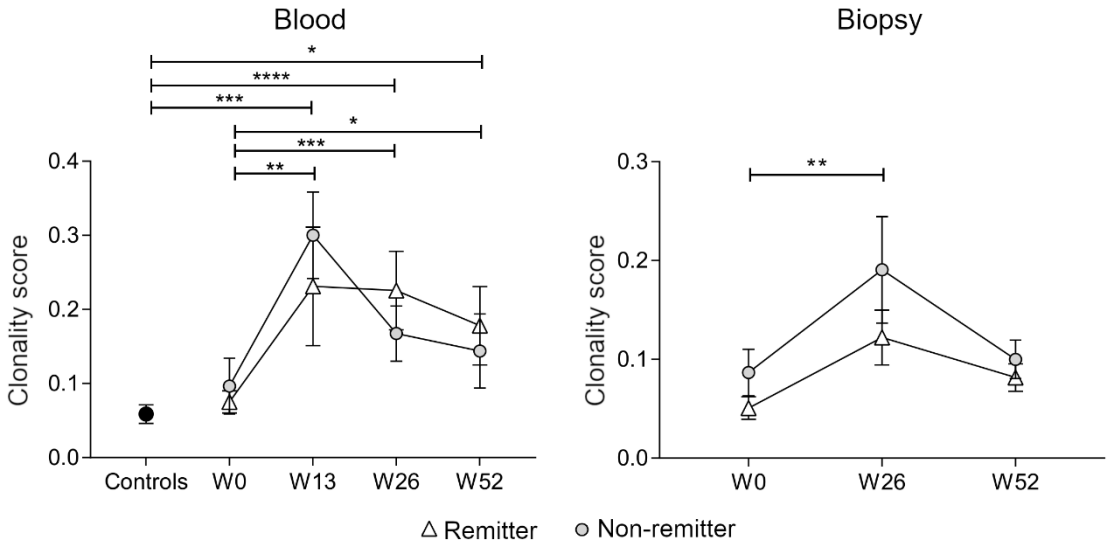


Figure 34. Clonality score increases after-HSCT. Clonality score in controls (n=10) and patients periphery (left, n=14, except W13 n=8) and biopsies (right, n= 10, except W0 n=9) categorized by remission achieved one year after HSCT. Remitters in blood: n=7, except W13 – n = 3; remitters in biopsy: n=5. Mean ± SEM; Mann-Whitney U test was used to compare between remitters and non-remitters at each time point. Wilcoxon signed-rank paired test was used to compare patients at different time points and a Mann-Whitney U test was used to compare patient and control samples * p. value < 0.05, ** p. value < 0.01, *** p. value < 0.001, **** p. value <0.0001

Following HSCT, the peripheral TCR clonality index significantly increased at 13 and 26 weeks compared to baseline (p.value = $7x10^{-3}$ and $1x10^{-4}$, respectively, Figure 34), indicating the high oligoclonality of these samples. This index was also altered in mucosal samples after HSCT, being significantly higher than baseline at week 26 after treatment (p. value = $3x10^{-3}$, Figure 34). This increase in biopsy clonality returned to baseline levels at 52 weeks. On the other hand, while clonality in the periphery tended towards baseline levels, it still remained higher at the same time-point (p. value = 0.04, Figure 34).

In summary, HSCT led to a decrease in total TCR clonotypes as well as a decrease in diversity at early time points. As the number of T cells recovered diversity was partially restored. This result was associated with an increase in the clonality index, reflecting a highly oligoclonal repertoire following HSCT. Remarkably, while remitters regained peripheral baseline levels of diversity at 1 year after HSCT, non-remitters diversity at 26 and 52 weeks after transplant remained significantly lower (Figure 33).

Changes in TCR repertoire are not related to control of Crohn's disease activity after HSCT

There is little in the literature on how TCRB sequences change over time in healthy individuals. Therefore, before tracking the clonotypes in patients undergoing HSCT, we determined the TCRB sequences present in the blood of 3 healthy non-IBD controls at two different time points: week 0 and week 3. With these results, we analyzed the similarity of the TCR repertoires, both by comparing the presence/absence of these clones and their abundance (Figure 35) and by using the Morisita-Horn (M-H) index (Figure 36). This index ranges from 0, representing two samples being completely different, to 1, meaning both samples have the same clonotypes with the same frequency.

Control	Shared clonotypes	W0 exclusive clonotypes	W3 exclusive clonotypes
C1	10,636	164,271	138,548
C2	12,009	97,559	103,752
C3	12,910	156,257	102,525

Table 9. Number of clonotypes shared at different time-points in the blood of 3 non-IBD healthy controls.

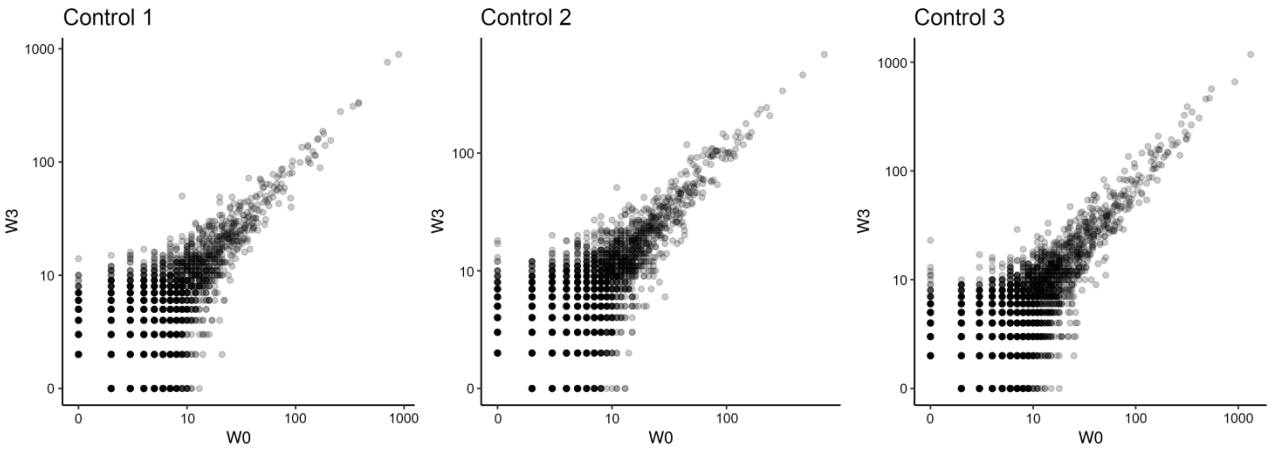


Figure 35. TCRB clonotypes and their abundances in two samples from three different control donors taken three weeks apart. Scatter plots showing the clonotypes (dots) and the number of sequences each clonotype had when present at week 0 (x axes) and week 3 (y axes).

As shown in Figure 35, control individuals had comparable repertoires in samples taken three weeks apart, sharing in similar abundance all of the highest frequency (and therefore more relevant) clonotypes. Furthermore, an M-H index comparing samples from the same control had close-to-one values (mean= 0.912, Figure 36). Nevertheless, a not-negligible amount of clones in the low frequency was not shared within three weeks (Figure 35, Table 9), suggesting that the probability of capturing low frequency clonotypes at any given time in this type of analysis is lower. Since over time all common clones in healthy individuals were the ones in the high frequency band, we set a frequency threshold for samples obtained from patients (described in the Materials and Methods section). It is important to mention that M-H indexes comparing inter-control samples were low (mean = $3.33x10^{-4}$, range 0-0.001, Figure 36). Indeed, we found no common clonotypes between any of the control samples sequenced.

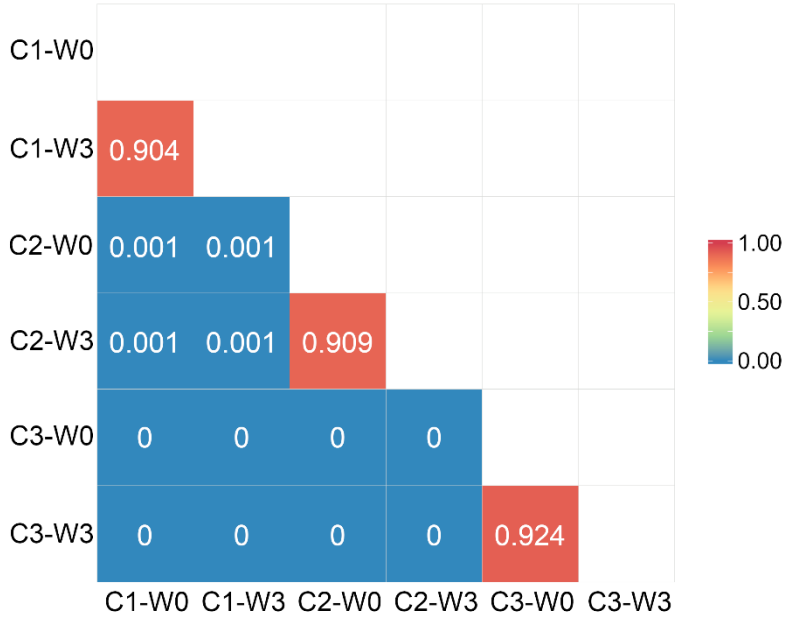


Figure 36. Morisita-Horn indexes comparing TCRB sequencing of blood control samples. Morisita-Index results of all possible combinations between control 1 (C1), control 2 (C2) and control 3 (C3) samples. The M-H index ranges from 0, representing two samples being completely different, to 1, meaning both samples have the same clonotypes with the same frequency.

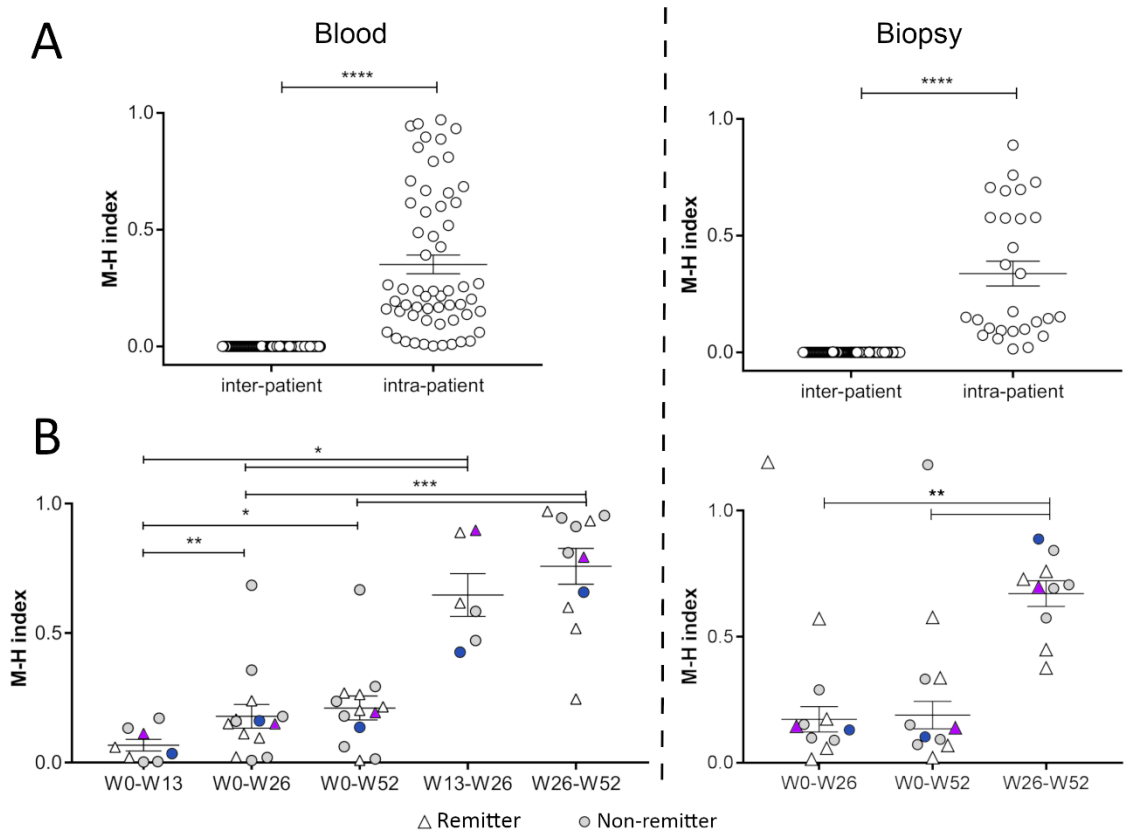


Figure 37. Changes in blood *TCRB* repertoire in patients with Crohn's Disease undergoing HSCT. **A**. Result of the Morisita-Horn index analysis of inter-patients and intra-patient samples for both blood (left) and biopsies (right). **B**. Similarity analysis between the *TCRB* repertoire of each patient following HSCT. M-H Index is represented for each patient and the mean ± SEM is shown. Remitters and non-remitters are identified with triangles and circles, respectively. P13 and P16 are highlighted in color (purple and blue, respectively) in order to identify them in Figures 37 and 38. Mann-Whitney U test was used to make comparisons between the inter- and intra-patient M-H indexes. Wilcoxon signed-rank paired test was used to compare the indexes at different time points. * p. value < 0.05, ** p. value < 0.01, *** p. value < 0.001, **** p. value <0.0001

Using this information, we analyzed the similarity of the TCR repertoires pre- and post-treatment in order to measure the percentage of surviving pre-existing clones. Notably, when we compared inter-patient samples, the mean M-H value in blood and biopsy was $1.81x10^{-5}$ (range 0-0.001) and $2.4x10^{-5}$ (range 0-0.001), respectively (Figure 37A), showing almost no common sequences between different individuals, in agreement with what we observed in control samples. Nevertheless, when we compared samples within each patient, including after-HSCT samples, the mean M-H value was 0.35 (range 0.001–0.97) and 0.33 (range 0.014-0.88), respectively (Figure 37A). Furthermore, only 29 out of a total of 69,168 clonotypes

found in high frequency in all patient samples were shared between two different patients (0.04%) and none was shared between more than 3 individuals. In short, each repertoire proved highly unique and there were no shared-by-all clonotypes. Hence, comparisons of the repertoires before and after HSCT were performed within each patient.

The overall similarity in the periphery between the TCR repertoire at baseline and at 13 and 26 weeks after HSCT was low (mean M-H = 0.06 and 0.17, respectively), indicating a large disruption in the TCR sequences. In contrast, a high degree of similarity (mean M-H=0.72) was observed between the repertoire at 26 and 52 weeks, showing the stability of the remodeled repertoire after HSCT (Figure 37B left panel). A similar result was obtained in the biopsies analysis, where the overall similarity between the TCR repertoire at baseline and at 26 and 52 weeks after HSCT was low (mean M-H = 0.17 and 0.18, respectively), and a high degree of similarity (mean M-H = 0.67) was observed between the repertoire at 26 and 52 weeks (Figure 37B right panel).

Finally, we identified high-frequency clones (using the threshold defined in Materials and Methods) in control and patient samples (Figures 38 and 39) and tracked baseline high frequency clones over time for each individual patient. In blood samples, an average of 86% of baseline high frequency clones were undetectable at any time point post HSCT, suggesting that they were significantly reduced or eliminated from the periphery. The rest of the baseline clones (averaging 14%, range 1.16-37.6) persisted with similar or greater frequencies at 13, 26 and 52 weeks after-HSCT regardless of response to treatment (Figure 38). In biopsies, an average of 79% of baseline high frequency clones was undetectable at any time point after HSCT, suggesting that they were eliminated from the tissue. The rest of the baseline clones (averaging 21%, range 5.4-69) persisted with similar or greater frequencies at 26 and 52 weeks post HSCT, regardless of response to treatment (Figure 39).

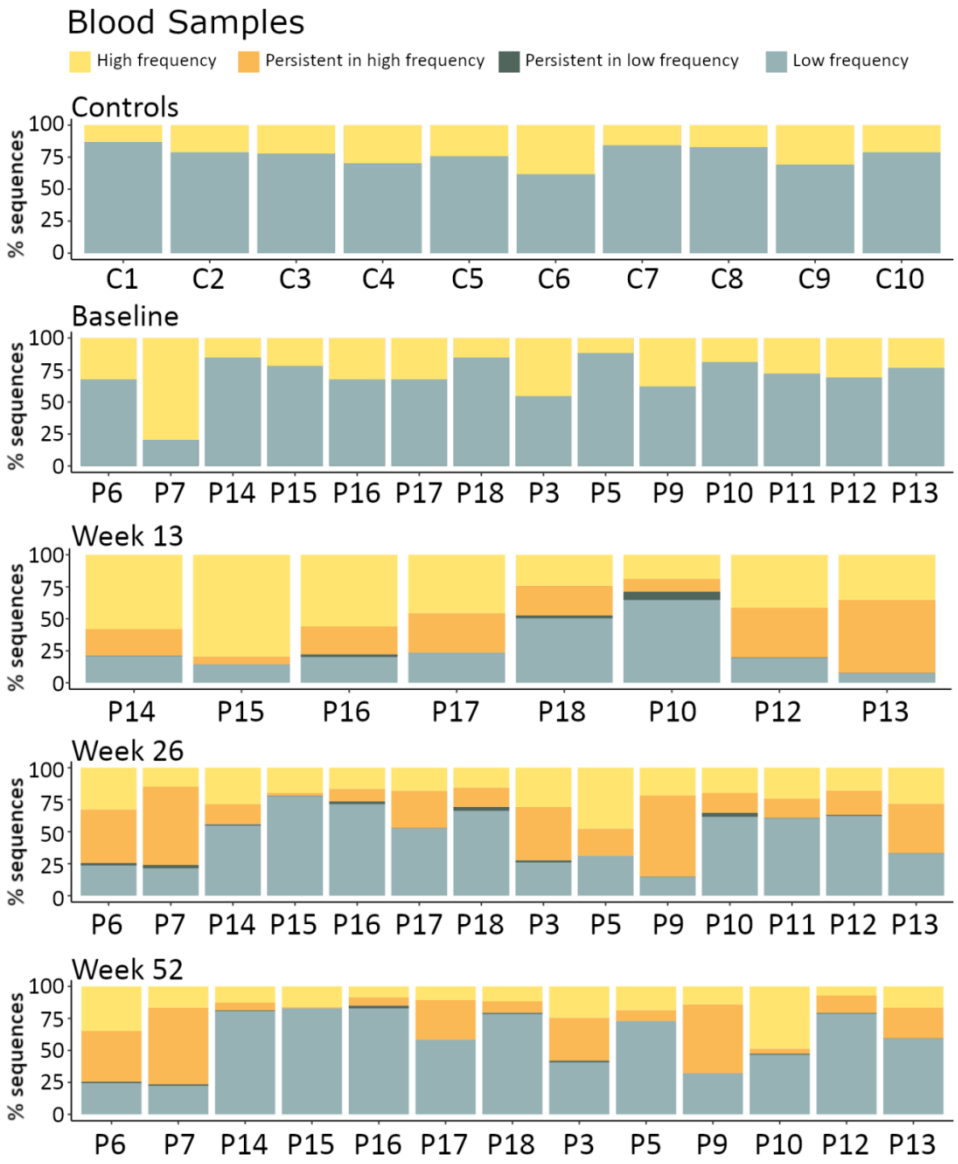


Figure 38. Percentage of high frequency clones in blood from control and patients with Crohn's disease undergoing HSCT. Each sample is presented as a bar with colors for clonotypes in high-frequency (yellow), low-frequency (grey). Persistent clonotypes at the different time-points after-HSCT (those that were present in high frequency at baseline) are shown in orange and green for high- and low-frequency, respectively.

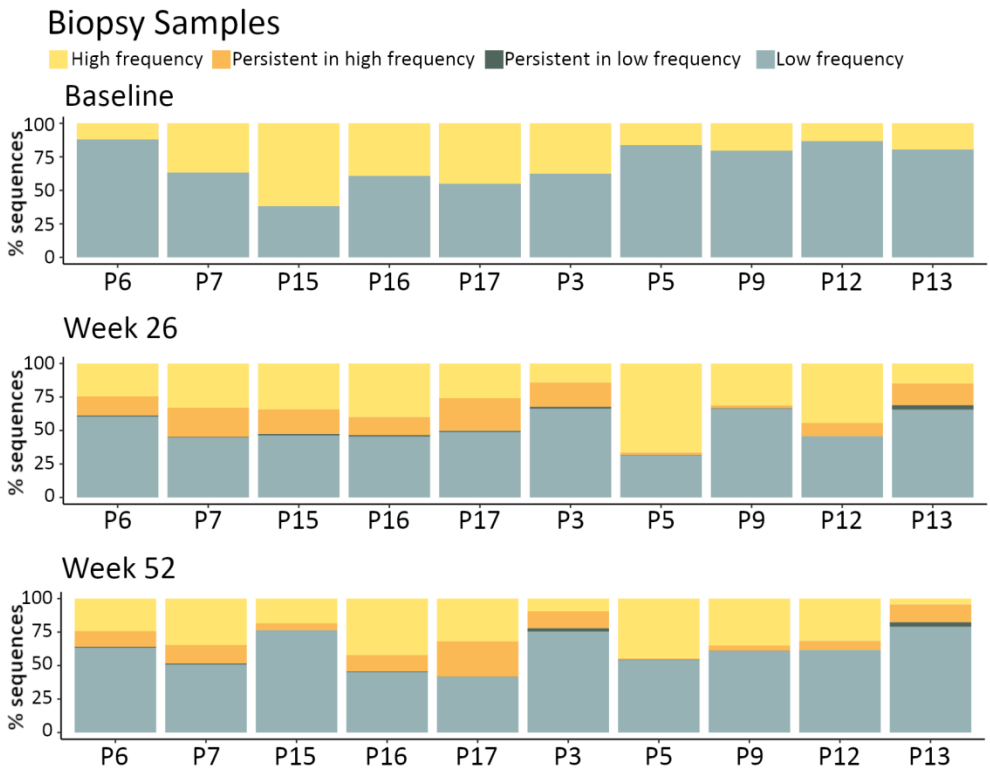


Figure 39. Percentage of clonotypes in the biopsy at different time points. Each sample is presented as a bar with colors for clonotypes in high-frequency (yellow), low-frequency (grey). Persistent clonotypes at the different time-points after-HSCT (those that were present in high frequency at baseline) are shown in orange and green for high- and low-frequency, respectively.

Figures 40 and 41 present donut plots for blood and biopsy, respectively, of two representative patients (also highlighted in Figure 37) with the top 10 clones shown in color. While the top ten clones mostly differed between pre- and post-transplant (bold clones), the majority of the blood 10 top clones at baseline were detected following HSCT, albeit at a lower frequency (shown with a plus symbol, Figure 40). After transplant, most of the top 10 clones were detected within the complete repertoire at baseline (marked with an asterisk, Figure 40). This effect was also present in biopsy samples, although it was not as pronounced (Figure 41).

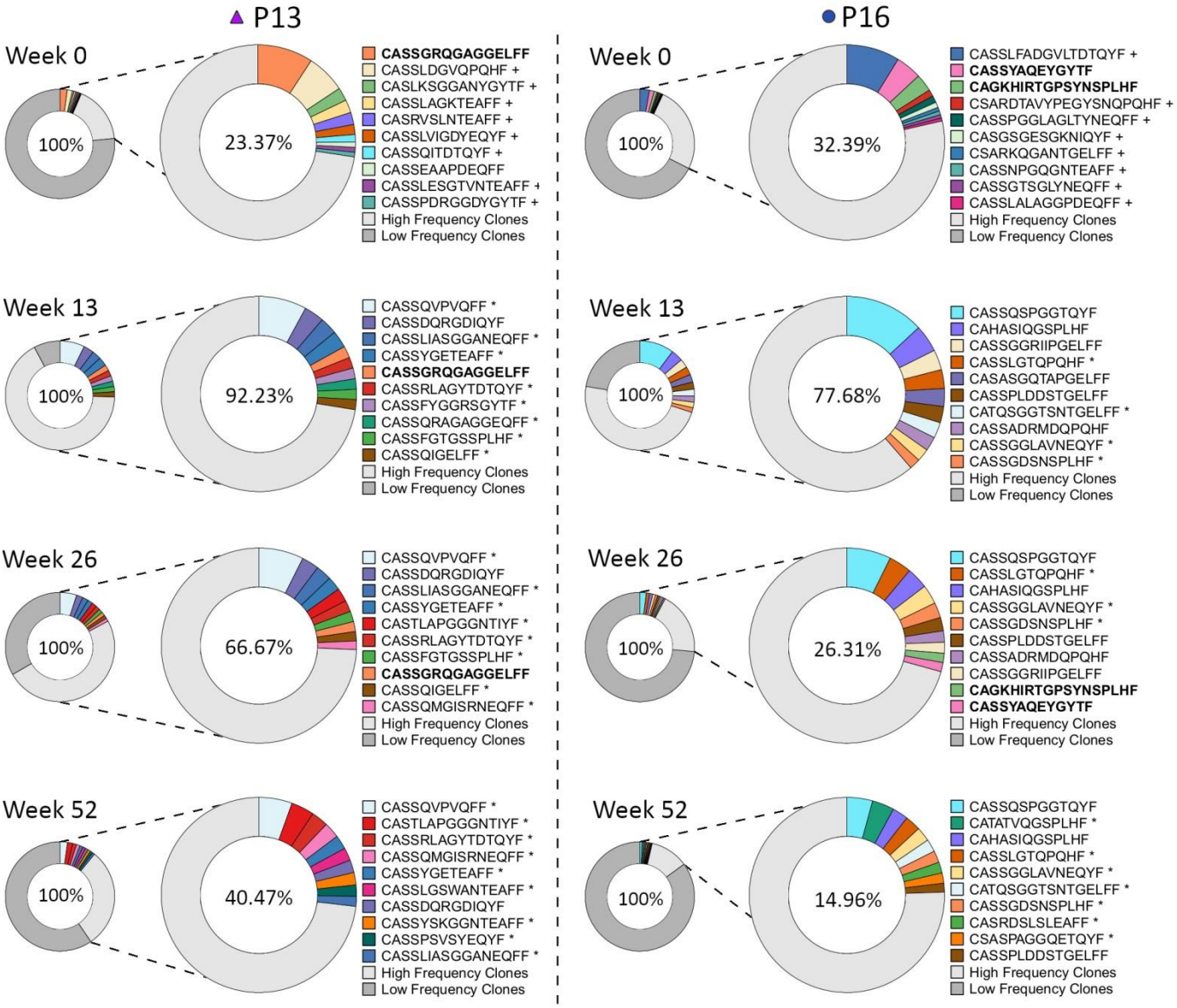


Figure 40. Donut plot representation of the distribution of high-frequency clones in the periphery before mobilization and transplant (week 0) and after-HSCT (weeks 13, 26 and 52). Two representative patients are represented (also highlighted in Figure 37) with the top 10 clones shown in color, a remitter (represented as a purple triangle) and a non-remitter (represented as a blue dot). Baseline clones that were detected following HSCT are marked with a plus symbol (+). After HSCT, clonotypes that were detected within the complete repertoire at baseline are marked with an asterisk (*). Clones that were present at top-10 frequency before and after HSCT are marked in bold.

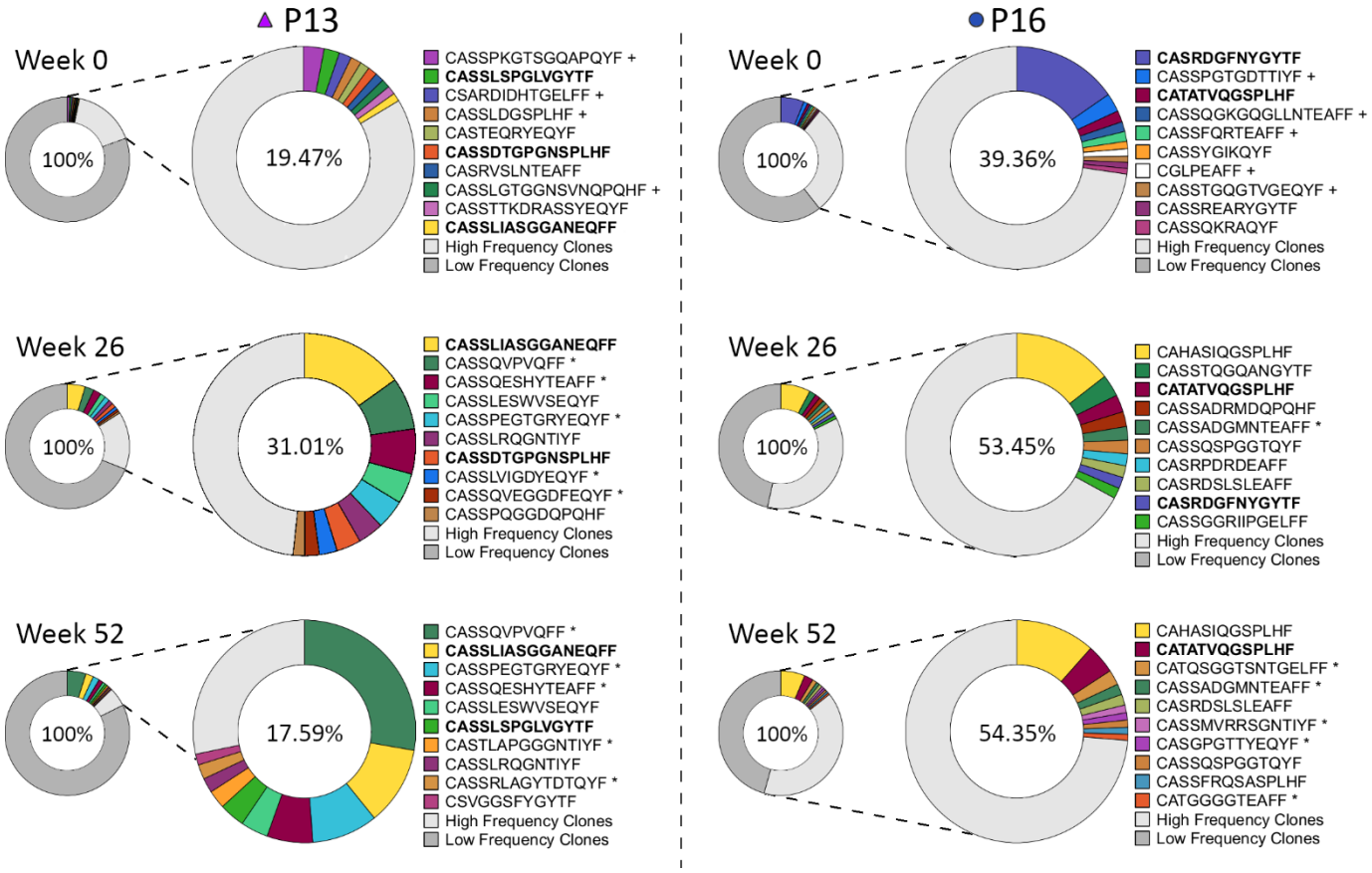


Figure 41. Donut plot representation of the distribution of high frequency clones in the biopsy before mobilization and transplant (week 0) and after-HSCT (weeks 26 and 52). Two representative patients are represented (also highlighted in Figure 37) with the top 10 clones shown in color, a remitter (represented as a purple triangle) and a non-remitter (represented as a blue dot). Baseline clones that were detected following HSCT are marked with a plus symbol (+). After HSCT, clonotypes that were detected within the complete repertoire at baseline are marked with an asterisk (*). Clones that were present at top-10 frequency before and after HSCT are marked in bold.

Overall, HSCT induced major changes in the pre-existing TCR repertoire in blood and biopsy samples. Indeed, a high percentage of clones present at baseline at high frequency (86% and 79%, respectively) were erased. However, regardless of response, we observed a not negligible number of clones that persisted after treatment.

HSCT induces remodeling of the B cell compartment

To investigate the specific clonal deletion or survival of B cells after HSCT, we sequenced the CDR3 region of the BCR (Immunoglobulin Heavy Chain (IGH) gene) in blood samples from 10 healthy controls and 14 patients with Crohn's disease undergoing autologous HSCT. Compared to TCR sequences, IGH templates in blood were markedly less abundant and their overall frequencies dramatically lower, which made tracking of specific clones in two sequential samples from the same donor impossible (Figure 42, Table 10)

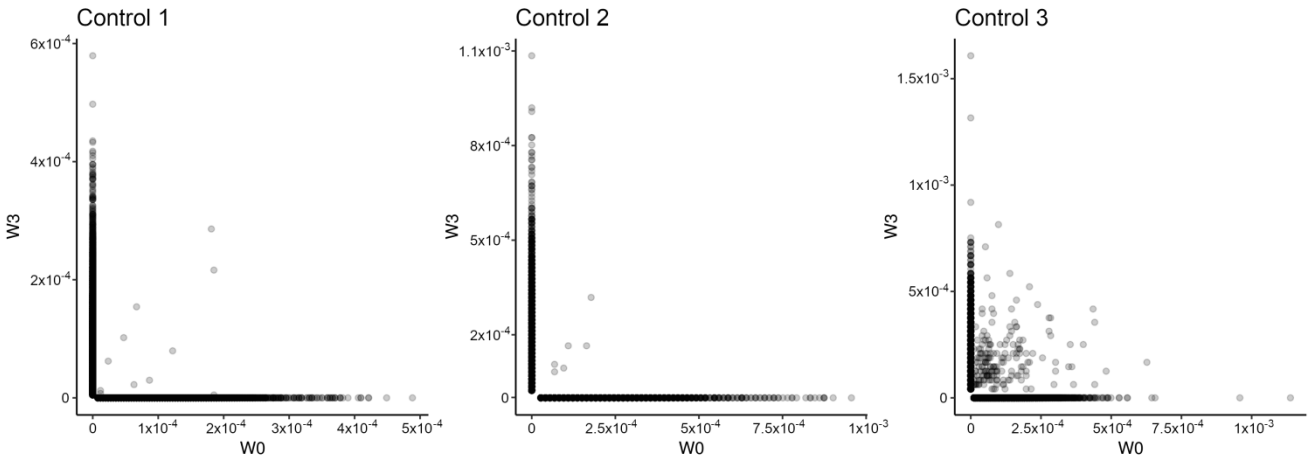


Figure 42. BCR clonotypes and their abundances in two samples from the same control donor three weeks apart. Scatter plots showing the clonotypes (dots) and the number of sequences each clonotype had when present at week 0 (x axes) and week 3 (y axes).

Control	Shared clonotypes	W0 exclusive clonotypes	W3 exclusive clonotypes
C1	11	12,248	13,790
C2	6	5,923	5,848
C3	179	11,012	5,790

Table 10. Number of clonotypes shared in two samples from the same control donor three weeks apart.

We could, however, determine the number of unique sequences (IGH clonotypes) and their overall frequency at baseline, as well as at weeks 26 and 52 after transplant. In agreement with the number of circulating B cells, the number of clonotypes was significantly higher at week 52 after HSCT compared to baseline and healthy age-matched controls regardless of the efficacy of the protocol (p. value = $4x10^{-3}$ and $2x10^{-3}$, respectively; Figure 43A).

In contrast to the marked clonal expansions observed within the T cell compartment, the clonality of the IGH repertoire was significantly lower at 1 year after HSCT compared to baseline (p. value = $7x10^{-3}$, Figure 43B), regardless of response to HSCT. This reflects the increase in the B cell repertoire due to expansion of the highly polyclonal naïve B cell compartment.

The renewal of the B cell repertoire was also reflected by the significant increase in the number of IGH sequences presenting no somatic hypermutations (SHMs), with a concomitant decrease at 26 weeks after transplant in the number of clonotypes with 1 or more SHMs (Figure 43C), representing activated B cells.[163]

Finally, we looked into family gene usage as a surrogate for BCR repertoire renewal. Crohn's disease patients at baseline and non-IBD controls showed comparable usage of the different VDJ family genes. After HSCT, we observed changes in V, D and J family gene usage. In particular, our analysis revealed a significant reduction in the usage of *IGHV03*, *IGHD02*, *IGHJ05* and *IGHJ06* at 26 and 52 weeks after HSCT compared to baseline (Table 11). Concomitantly, we observed an increase in *IGHV01*, *IGHV04*, *IGHV05*, *IGHD06*, *IGHD07*, *IGHJ02* and *IGHJ03* gene usage at those same time points (Table 11).

Overall, we can conclude that the B cell repertoire becomes more diverse after HSCT regardless of the response to treatment. Remodeling of the B cell receptor repertoire can be inferred from the massive expansion of naïve cells (as seen by flow cytometry, transcriptional analysis and SHM analysis) and the changes in family gene usage. Nonetheless, given the low frequency of the clonotypes detected in blood, the possibility that the remaining B cell clones survived after HSCT, as was the case with T cells, cannot be excluded.

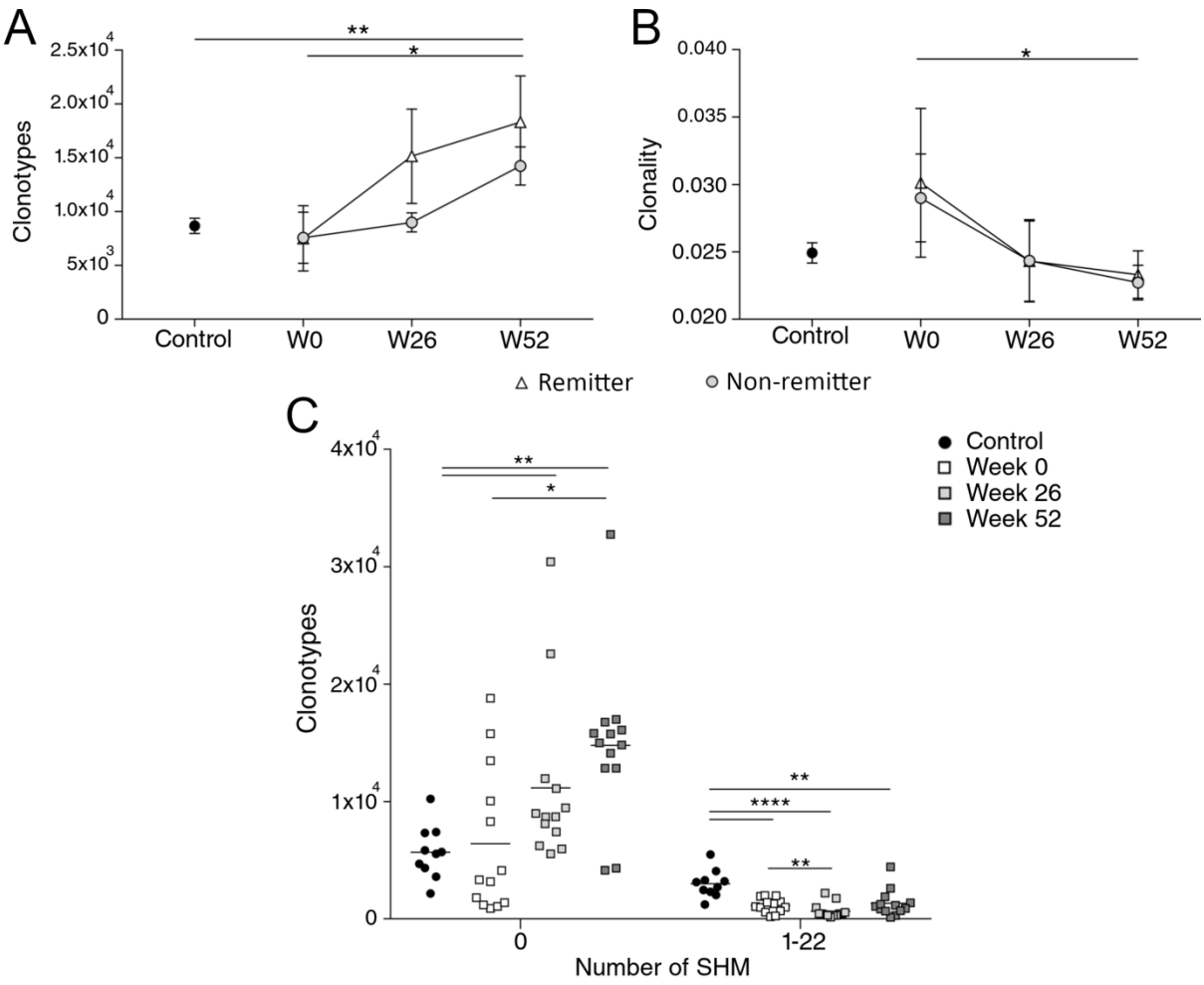


Figure 43. Analysis of whole blood IGH sequences in Crohn's disease patients undergoing autologous HSCT. **A**. Number of clonotypes, defined as unique sequences, in Crohn's disease patients (n=14) at baseline (W0) and at weeks 26 and 52 after treatment. Non-IBD controls (n=10) are included for comparison. Patients were categorized by response (n=7, per group). **B**. Clonality score (mean ± SEM) for controls (n=10) and Crohn's disease patients, categorized as remitters (n=7) and non-remitters (n=7). **C**. Number of BCR clonotypes based on the number of somatic hypermutations (SHM) in the CDR3 IGH sequences. Paired Wilcoxon signed-rank test was used to test differences between various time points and a Mann-Whitney U test was used for patients-controls comparisons. *p.value < 0.05, **p.value < 0.01, ***p.value < 0.001, **** p.value<0.0001

Family	Controls	Week 0	Week 26	Week 52
IGHV01	16.88 ± 3.86	19.00 ± 2.55	20.57±2.14 * ††	20.10 ± 2.19 *
IGHV02	3.95 ± 0.88	4.42 ± 0.86	4.69 ± 0.97	4.77 ± 0.97
IGHV03	52.85 ± 4.76	50.89 ± 3.53	48.63 ± 3.20* ††	48.34 ± 3.06 * ††
IGHV04	20.31 ± 2.37	20.44 ± 1.95	20.78 ± 1.54 †	21.39 ± 1.50 †
IGHV05	4.59 ± 1.49	3.71 ± 1.24	3.87 ± 0.99 †	4.08 ± 0.96 †
IGHV06	0.93 ± 0.22	0.91 ± 0.21	0.91 ± 0.23	0.90 ± 0.22
IGHV07	0.38 ± 0.68	0.38 ± 0.55	0.42 ± 0.52	0.30 ± 0.45
IGHD01	8.15 ± 1.32	8.10 ± 1.10	8.20 ± 1.37	8.19 ± 1.25
IGHD02	13.13 ± 1.76	13.10 ± 2.22	11.15 ± 1.88 ** †††	11.11 ± 1.56 **†††
IGHD03	32.49 ± 2.58	33.61 ± 1.43	34.12 ± 1.82	34.51 ± 1.78 *
IGHD04	8.01 ± 1.04	7.40 ± 0.79	7.59 ± 1.02	7.83 ± 0.99
IGHD05	7.99 ± 0.98	8.28 ± 0.99	8.51 ± 1.10	8.73 ± 0.81
IGHD06	17.14 ± 1.53	17.43 ± 2.47	19.83 ± 1.10 *** ††	19.04 ± 0.93 **†
IGHD07	0.88 ± 0.20	0.96 ± 0.33	1.37 ± 0.42 ** ††	1.28 ± 0.41 ** ††
IGHJ01	1.24 ± 0.24	1.44 ± 0.34	1.61 ± 0.24 **	1.54 ± 0.27 *
IGHJ02	2.72 ± 0.65	2.88 ± 0.59	3.36 ± 0.53 ** †	3.34 ± 0.50 ** †††
IGHJ03	9.69 ± 1.95	10.82 ± 2.83	13.74 ± 2.75 *** †††	13.63 ± 2.71 *** †††
IGHJ04	44.35 ± 3.45	43.70 ± 2.31	45.27 ± 1.75	45.29 ± 1.53
IGHJ05	12.05 ± 1.08	11.28 ± 1.04	10.04 ± 0.78 *** †††	10.36 ± 0.88 ** ††
IGHJ06	26.26 ± 5.42	26.58 ± 4.68	23.77 ± 3.66 ††	23.64 ± 3.44 †††

Table 11. IGH family gene usage at baseline and during follow-up. Each column of values shows the mean ± SEM. *comparison to controls; *p. value < 0.05, ** p. value < 0.01, *** p. value < 0.001; †comparison to W0; †p. value < 0.05, †† p. value < 0.01, ††† p. value < 0.001

IgG levels in serum remain unchanged after HSCT in Crohn's disease patients

HSCT impacts B cell populations in blood, which may affect antibody production. Hence, we measured changes in serum antibody concentrations following HSCT. Total serum IgA and IgM significantly decreased in patients following HSCT, while the concentration of total IgG remained unchanged up to 1 year after immune ablation (Figure 44).

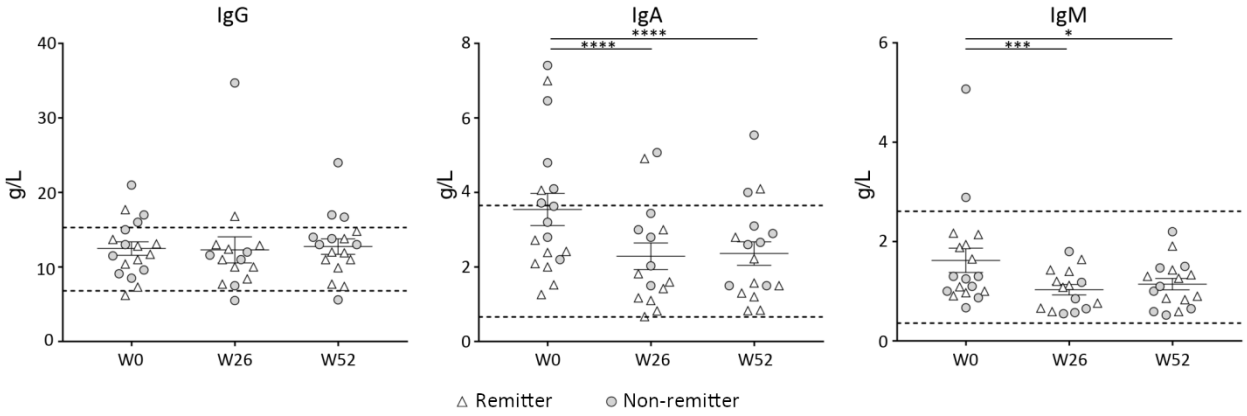


Figure 44. Antibody levels in serum remain unchanged after autologous HSCT in Crohn's disease patients. Serum IgG, IgA and IgM concentrations (g/L; mean ± SEM) at baseline (W0) and after HSCT (W13, W26 and W52) in Crohn's disease patients (n=18). Dotted line represents the serological protection level. n = 18; *p. value < 0.05, ** p. value < 0.01, *** p. value < 0.001; significant by Wilcoxon signed-rank Paired test.

IgG levels against tetanus toxoid (TT), rubella, varicella zoster, epidemic parotitis, measles and pertussis vaccines were measured in theserum of all patients from week 0 up to one year after transplant (Figure 45). During this period, the majority of patients maintained the same level of serological protection against these pathogens they had at baseline. For TT, patients are re-vaccinated about 6 months after transplant, and hence, specific antibodies surged at week 52 (Figure 45A)

We also measured IgG ASCA[35] in the same group of patients (Figure 46). About 50% of patients presented ASCA levels above the established normal threshold at week 0. Changes in ASCA after transplant varied markedly at the individual level. Remarkably, for those patients with detectable ASCA levels before transplant, a decrease in ASCA following HSCT did not correlate with disease improvement (Figure 46).

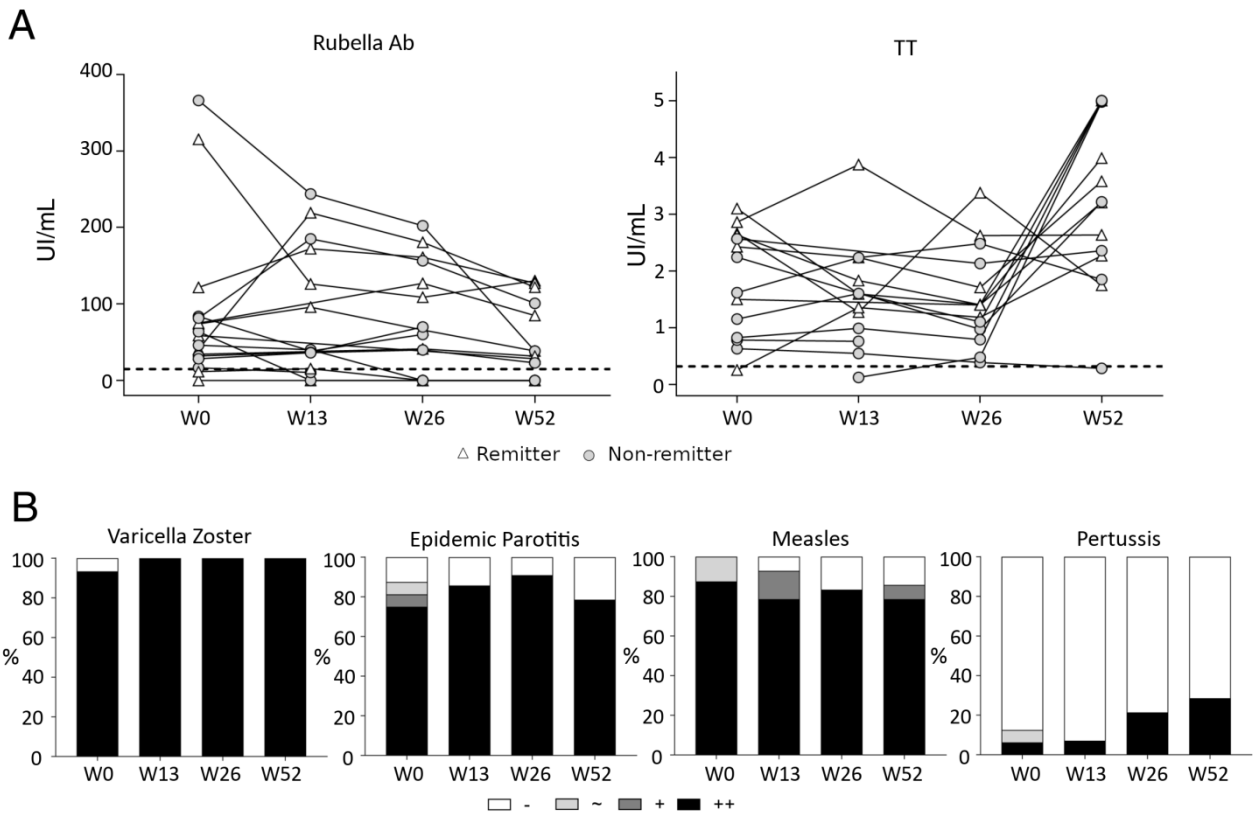


Figure 45. Serum IgG antibody levels against vaccines in Crohn's disease patients undergoing HSCT. **A**. Antibodies against Rubella (U.I./mL) and tetanus toxoid (TT) present in serum (n=18) were determined by ELISA at baseline (W0) and after HSCT (W13, W26 and W52). Dotted line represents the serological protection level. **B**. Percentage of patients based on their protection levels against different pathogens. Levels of serological protection were detected by ELISA in our Crohn's disease patient cohort (n=18) at baseline and after HSCT.

We measured IgG concentrations against *Escherichia coli* flagellin proteins (Fla2, FlaX) and a predicted lipoprotein (YidX). Both are bacterial proteins previously associated with Crohn's Disease[36, 37] and the results proved similar (Figure 46). These results suggest that while B cells and antibody responses are impacted by HSCT, these parameters do not correlate to treatment efficacy.

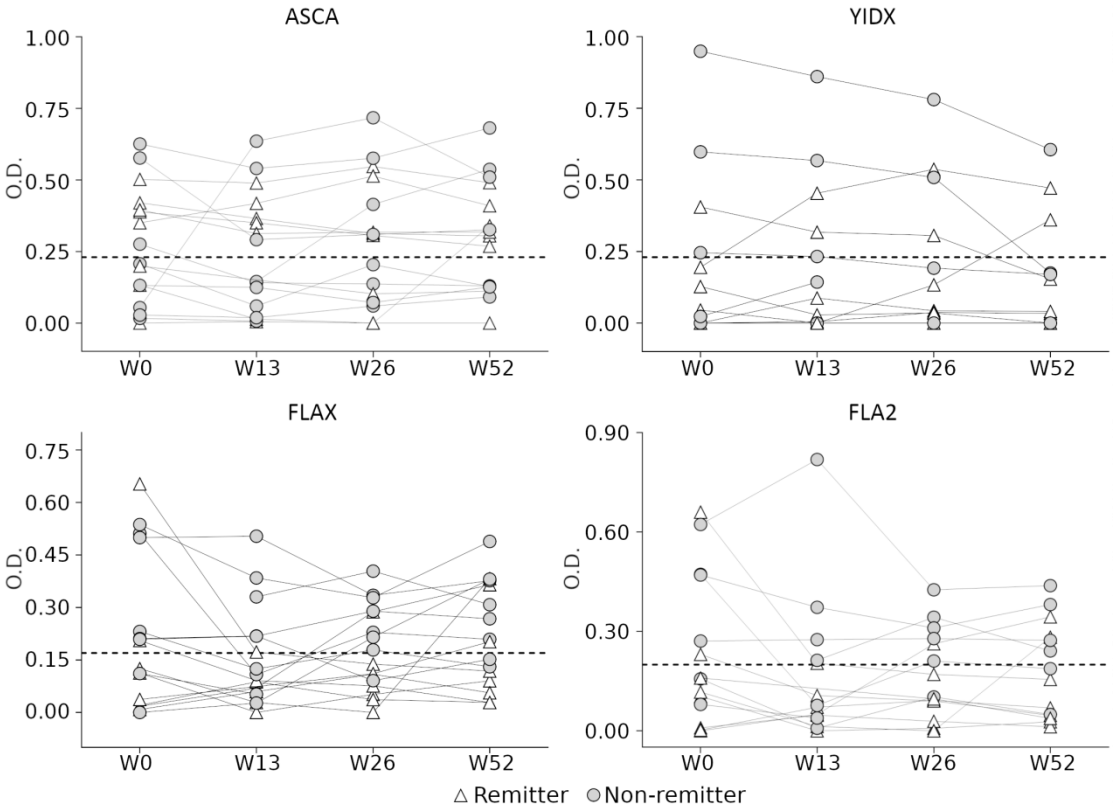
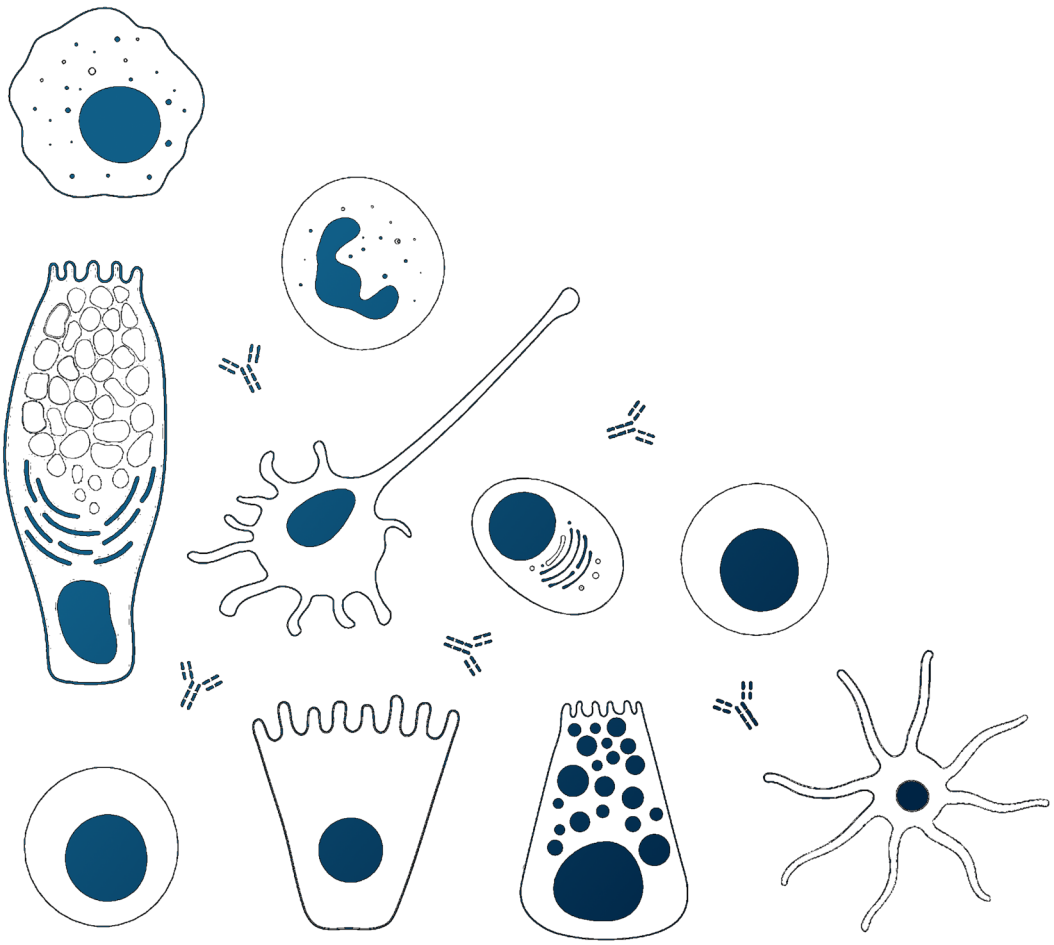


Figure 46. Serum IgG antibody levels against microbial antigens in Crohn's disease patients undergoing autologous HSCT. Serum IgG antibodies against anti-*Saccharomyces cerevisiae* (ASCA), E coli-derived seroreactive protein YidX, and flagellins Flax and Fla2 at baseline (W0) and after HSCT (W13, W26 and W52). Values represent extinctions at optical density (O.D.) 620 obtained by ELISA. The dotted line represents the threshold established for seroreactivity (mean O.D.) in a group of healthy controls.

Discussion



The mechanisms that lead to the control of a dysregulated immune response following autologous HSCT are still not entirely clear. The hypothesis is that the conditioning regime eliminates committed pathogenic lymphocyte clones, leading to de novo generation of immune cells that re-establish tolerance.[145, 164] Indeed, autologous HSCT has been shown in the context of various immune-mediated diseases to remodel the peripheral immune system, as evidenced by the regeneration of naïve B cells[152, 165], thymic reactivation[146, 152, 166, 167], renewal of the TCR repertoire[146, 152, 167] and regeneration of the Treg cell compartment.[150, 151]

Nonetheless, specific studies exploring immune reconstitution in patients with Crohn's disease following autologous HSCT are almost nonexistent. A study from Clerici and collaborators did analyze peripheral immune changes in a cohort of 7 patients with Crohn's disease undergoing autologous HSCT.[136] In particular, the authors reported variations in the percentage of peripheral blood monocytes that produced TNF-α and IL-10 at 6 and 12 months after autologous HSCT. In a subset of 3 out of 4 patients defined as "full responders" (patients that achieved endoscopic remission 6 months after transplant), they observed a transient increase in the percentage of circulating Treg cells during the first 6 months. Besides this limited characterization, no study to date has monitored the immune changes that take place in the peripheral blood of patients with Crohn's disease receiving autologous HSCT. Furthermore, to our knowledge no data was available on the cellular and molecular changes that take place in the mucosa of these patients.

Peripheral cellular composition assessed by flow cytometry in our cohort clearly shows that 3 months after severe immune ablation and HSCT, the $CD4^+$ T cell compartment remains compromised, while the $CD8^+$ T and the B cell subsets have completely recovered (Figure 16). Moreover, and in agreement with the existing literature[168], peripheral naïve cells within the $CD4^+$ T cells remained almost completely depleted at the 3-month follow-up time point, but then recovered to baseline levels by 1 year after transplant. We further confirmed these findings by peripheral blood transcriptomics. Indeed, we observed down-regulated T cell pathways at week 13 and up-regulated B cell pathways at week 26 (Figure 16). Overall, this data supports the notion that following HSCT Crohn's disease patients

reconstitute the naïve T cell compartment through mechanisms of homeostatic proliferation and/or thymic output.

In contrast to the naïve compartment, circulating memory T cells, while significantly reduced compared to baseline at week 13, were not completely depleted at that time, suggesting that memory clones may survive chemotherapy or may be reintroduced in the graft. Despite this partial persistence, the $CD4^+$ memory compartment showed a delayed recovery, as seen by both flow cytometry (Figure 17) and gene expression (Figure 20), in agreement with the existing literature.[169] On the other hand, $CD8^+$ cells rely mostly on the peripheral expansion of mature cells, and not on thymic function and, as we confirm here, are rapidly replenished after transplant.[169, 170] This disconnect between replenishment of the CD4 and CD8 compartments after HSCT has also been observed in patients with multiple sclerosis.[143]

The rapid recovery and later expansion of the B cell compartment we observed after HSCT was also in agreement with previous findings[152]; however, the persistence of serum IgG antibody titers was unexpected. Previous studies have shown a decrease in circulating immunoglobulins following high-dose chemotherapy and autologous $CD34^+$ reconstitution[171], with serum IgA and IgM levels still significantly decreased 9 months after reconstitution. Indeed, we observed a significant decrease in total serum IgA and IgM immunoglobulins at 6 and 12 months of follow-up. This reduction, however, was also detected in non-remitters. On the other hand, IgG levels remained unchanged after HSCT, and the protection afforded by common vaccines based on antibody serum levels was preserved after HSCT, in contrast to what has been previously reported.[152] It is important to note that much of the evidence on antibody titers after transplant is based on patients who received more intense conditioning regimes[171], raising the possibility that the less aggressive protocol used for immune-mediated diseases does not require massive re-immunization afterwards. Nevertheless, this needs to be confirmed in a larger patient cohort.

There are few studies analyzing the changes in cytokine serum levels after HSCT. Sun *et al.* observed an increase in serum levels of IFN-γ, TNF-α, and IL-10 in multiple sclerosis patients 3 months after autologous HSCT.[172] After 12 months these cytokines decreased to baseline levels. In addition, they observed that IL-12 serum levels consistently decreased following HSCT in all patients. To our knowledge, there is no previous report on serum cytokine changes in Crohn's disease patients after HSCT. In our cohort, serum concentrations of pro-inflammatory cytokines such as IL-6 and IL-12p70 are significantly decreased shortly after HSCT and baseline levels are recovered at one year. On the other hand, the concentration of CXCL10 is significantly increased at week 13 after HSCT and normalized by week 26. None of the other 35 measured cytokines showed significant changes in serum concentrations. Remarkably, the modulations in IL-6, IL-12p70 and CXCL10 were not related to achieving disease remission at 6 months or 1 year after HSCT. Altogether, our data suggests that serum cytokine concentrations may not be used as reliable surrogate markers to monitor changes in disease after HSCT.

On the other hand, our study shows that monitoring the overtime recovery of the peripheral immune compartment after HSCT provides some measure of the extent of immune depletion, but does not correlate with endoscopic remission after HSCT therapy. This strongly suggests that lack of efficacy in some patients may not be related to insufficient immune depletion. Nonetheless, circulating cells may not accurately reflect the extent of immune depletion in the involved tissue after autologous HSCT, which might be of major relevance to understanding how remission is achieved after therapy. Hence, we focused our analysis on the intestines of Crohn's disease patients undergoing transplant and compared the transcriptional signatures of mucosal biopsies before and after HSCT.

Besides an in-depth study of the immune changes taking place in the peripheral compartment, this thesis delved into the cellular and molecular mechanisms regulated in the intestine following HSCT. Given the limited number of total biopsies that could be obtained from these patients, performing cellular phenotyping (by means of FACS analysis) would have only provided information on a limited number of cells and markers. Instead, we chose a whole genome transcriptional approach to investigate the complete transcriptome in response to

HSCT, which generated information on the expression of over 17,000 protein-coding transcripts per sample. Differential expression gene analysis of these biopsies revealed over 2,000 genes that were significantly regulated in remitting patients at one year compared to samples obtained at baseline. This signature comprised hundreds of genes that were also modulated by anti-TNF therapy and included cytokine-mediated inflammatory pathways, genes expressed by infiltrating immune cells, such as granulocytes and activated macrophages, as well as tissue remodeling and regenerating pathways that have been extensively described as being modulated in response to conventional treatment.[173, 174] Our analysis clearly shows that in this group of Crohn's disease patients (50% of our study cohort), HSCT was able to drive the mucosal healing that is associated with profound molecular changes characteristic of disease remission.

Remarkably, about 50% of the signatures of HSCT-remitters was not shared by anti-TNF remitters. This HSCT-exclusive signature turned out to be primarily comprised of T-cell-related genes, suggesting profound changes in the mucosal T cell content and phenotype following HSCT. This observation was further confirmed by deconvolution analysis. This computational approach looks at whole genome transcriptional signatures from complex tissues such as the intestinal lamina propria to enumerate cell subsets present at different time points. Using this methodology, we characterized and inferred changes in tissue cell composition following HSCT. While achieving endoscopic remission in response to HSCT or anti-TNF therapy was associated with changes in the neutrophil and M1 macrophage proportions in biopsies, significant changes in other immune cellular subsets, such as naïve B cells or certain populations of T cells, were only observed after transplant.

Indeed, our data suggests that HSCT achieved a significant reduction in total T cell mucosa content based on the significant down-regulation of genes such as *CD3E* and *CD28*. This profound effect on the overall T cell content appears to be more pronounced in stem cell transplant therapy and is likely induced by the ablative treatment. This effect, in contrast to the depletion of T cells in the peripheral blood, was not seen in non-remitters to HSCT, strongly suggesting that depletion of T cells in the mucosa is linked to the efficacy of this treatment. One possibility requiring further study is whether HSCT, but not anti-TNF, can act on the

tissue-resident T cell memory compartment, which constitutes a predominant T cell subset in healthy mucosal tissues.[175] These non-migratory T cells are poorly characterized in the context of Crohn's disease and have been described as acquiring *CD69* expression, while losing *S1PR1* upon tissue entering, as well as the integrins αE (*CD103*) and α1 (*ITGA1*).

Nevertheless, this hypothesis would need to be validated by single-cell approaches, such as single-cell RNA sequencing. This methodology could provide information on cell types that are resistant to therapy in non-remitter patients; therefore, it might further our understanding of the mechanisms involved in achieving remission after autologous HSCT. Furthermore, the identification of cell-specific effects of a potentially curative treatment would bring some light on the discovery of specific targets for potentially less aggressive and more effective therapies.

We hypothesized that sufficient T cell depletion within the intestine (achieved by the conditioning regime) may be one of the hallmarks of HSCT efficacy in Crohn's disease patients and could determine the treatment's success. In order to explore this hypothesis, we performed in-depth TCR repertoire characterization. This analysis was performed by sequencing the specificity-determining CDR3 sequences of the receptor's beta chain. In a previous study, Kelsen *et al.* identified TCRD clonotypes to assess the expansion and possible malignant transformation of γδ-T cells after anti-TNF treatment.[61] They collected blood from 46 Crohn's disease patients receiving either infliximab or adalimumab and examined changes in γδ-T cells at early points during anti-TNF therapy by flow citometry and clone sequencing. Their results show that 90% of the γδ-T cells were Vδ2, suggesting the existence of a predominant γδ-T cell clone after anti-TNF therapy. Nevertheless, the technique used in this study did not allow for the sequencing of the complete CDR3, and it was not possible to determine the actual clonality and diversity of the γδ-T cell repertoire.

In a more recent study, Allez in collaboration with our own group[176] used the same *TCRB* CDR3 sequencing technology described in this thesis, which is capable of sequencing the entire CDR3 region. The CDR3 sequences in mucosal samples

were analyzed before and six months after surgery in a prospective multicenter cohort that included 57 Crohn's disease patients. The objective was to assess the impact of the TCR repertoire on post-operative recurrence in Crohn's disease. The study showed that smoking was linked to a reduction in the repertoire diversity and an increased proportion of T cell clonal expansions, which was associated with a high risk of postoperative endoscopic recurrence.

Anti-TNF has been shown to partially modulate the mucosal T cell repertoire, acting primarily on highly expanded clones.[129] Doorenspleet *et al.* sequenced the mucosal TCRB repertoire in the colon and ileum of 19 Crohn's disease patients and 11 controls using a next-generation sequencing approach.[129] They compared patients with Crohn's disease and controls, and showed that clonal expansions were present in the intestines of both groups. Nevertheless, Crohn's disease patients had remarkably larger expansions. Their study also showed that one-third of the repertoire was common between inflamed and non-inflamed tissue, as well as between the colon and ileum.[129] Regarding response to anti-TNF, while they showed that anti-TNF can modulate the T cell infiltrate, a non-negligible part of the repertoire in the mucosa of Crohn's disease patients persisted regardless of the response to this biologic.[129]

In our HSCT cohort, we observed that even in patients who achieve endoscopic remission after HSCT, remodeling of the TCR repertoire remains incomplete in both peripheral and mucosal samples. A non-negligible number of baseline T cell clonotypes survived the conditioning regime and were detected even at the earliest time point we analyzed (13 and 26 weeks post-HSCT in the periphery and in biopsies, respectively). Indeed, previous studies of peripheral blood in patients with multiple sclerosis had already concluded that complete TCR renewal after HSCT was not needed to induce disease remission. Multiple sclerosis patients had sustained disease remission after HSCT despite the fact that pre-existing high-frequency T cell clones remained present in the blood after transplantation.[143] Nonetheless, these early studies did not analyze the rates of clonal deletion in non-remitters.[143, 146] Here, we show for the first time that non-remitters have comparable degrees of immune reconstitution, including measures of T and B cell remodeling.

We also confirm here that severe immune T cell depletion after HSCT is followed by expansion of the remaining clones at early time points in both blood and mucosal samples. This had previously been described in the periphery,[146, 177, 178] even in patients receiving T-cell-depleted CD34⁺ autologous grafts, which suggests that the expanded T cell clones may represent surviving effector memory cells.[179] Oligoclonal expansions following HSCT in patients with multiple sclerosis have been shown to be primarily contained within the CD8⁺ compartment, while the reconstituted CD4⁺ compartment would be primarily, but not exclusively, comprised of new clones.[143] This data suggests that reconstituted CD8⁺ compartment is mostly created by clonal expansions of pre-existing cells, which would fit with their rapid recovery after transplant. Oligoclonal CD8 expansions following HSCT cells could bias peripheral immune reconstitution, by competing for space and actually limiting the potential for re-expansion of self-antigen specific-memory T cells.[145] While our analysis cannot distinguish between CD4- or CD8-expressed TCRs, based on the published literature we speculate that a majority of the persisting expanded clones after transplant are CD8⁺ T cells. Furthermore, we confirmed the observation by Allez *et al.* that there were no identical CDR3 sequences shared by more than one patient. This shows that there are no universal clones playing a key role in the persistence of the disease.[176]

In striking contrast to T cells, B cells recover immediately after HSCT and undergo a marked expansion both in the blood and intestine. Remarkably, the expansion of naïve B cells within the mucosa of patients in remission after HSCT closely correlated with the changes observed in the peripheral blood of these patients, and as mentioned above, was not observed in response to anti-TNF treatment. The biological effects and consequences of this B cell expansion are difficult to ascertain. However, based on preliminary data on both blood and tissue samples at 2 years after transplant, this expansion is temporary and may merely reflect the enlargement of this compartment following the severe aplasia induced by the conditioning protocol.

To gain some further insight into the remodeling of the B cell compartment, we also sequenced the BCR CDR3 segment in our patient cohort. In contrast to the T cell compartment, the B cell compartment cannot be tracked by sequencing of the

receptor. IGH templates in the blood were markedly less abundant and their overall frequencies dramatically lower. This fact made impossible the tracking of specific BCR clones in two sequential samples from the same healthy donor. Therefore, we used a different approach utilitzing the number of SHM as a surrogate for naïve/memory cells, and the classification of the family gene usage as a surrogate for BCR renewal. Hence, remodeling of the BCR repertoire was inferred from the massive expansion of naïve cells (as seen by flow cytometry, transcriptional analysis and SHM analysis). Nonetheless, given the low frequency of the clonotypes detected in blood, the possibility that the remaining B cell clones survived after HSCT, as was the case with T cells, cannot be excluded. Similar to T cells, the B cell compartment in both remitter and non-remitter patients is regenerated to an equal degree. This further refutes the contention that insufficient clonal deletion results in a lack of response to HSCT in Crohn's disease patients.

Overall, insufficient T and B cell remodeling after HSCT does not seem to explain the lack of efficacy in some patients. However, our data shows that non-remitters may instead present a pre-existing constitutive defect in their TCR diversity. To date, only the study by Muraro *et al.*[143] investigated the immune mechanisms underlying the lack of response to HSCT in autoimmunity. In that study, in which 20 of 24 patients achieved a complete response after HSCT, they reported a trend towards decreasing repertoire diversity at 2 months following HSCT in the 4 patients who failed to meet the primary endpoint following transplant. The authors hypothesize that this lack of diversity reconstitution may be related to the presence of oligoclonal auto-reactive cells. Indeed, our data shows that significantly lower TCR diversity is common in patients who do not benefit from the procedure, and that this lack of diversity is not "corrected" by intense immune remodeling following HSCT.

This thesis provides the first description of peripheral cell depletion and reconstitution following immune ablation and autologous HSCT in a group of patients with Crohn's disease. Importantly, this is to our knowledge the first time that immune reconstitution following HSCT has been compared in patients who benefit from HSCT and those who do not. While previous studies in other autoimmune diseases have suggested that peripheral immune renewal or "re-

education" might explain the induction of remission, we demonstrate here that this change alone does not guarantee it. We show that the procedure induces comparable changes in all the studied parameters in patients regardless of response, strongly suggesting that lack of efficacy is not due to insufficient immune ablation.

These results, together with the efficacy and safety studies developed by our group[130, 131], have led to the design of a new autologous HSCT regime. Since we observed that lack of efficacy is unrelated to insufficient immune ablation, a new protocol has been established that eliminates altogether the use of cyclophosphamide during the mobilization phase. The main objective of the new regime is to decrease treatment-related morbidity without affecting overall efficacy. Therefore, mobilization of HSCs is induced by G-CSF alone (12-16μg/kg/daily for 5 days). Patients who fail to achieve mobilization of more than 2 million $CD34^+$ cells (expected to be around 20% of the population based on previous literature[180]) are treated with plerixafor.

Plerixafor (AMD3100, Mozobil®) is a specific antagonist of the chemokine receptor CXCR4, which was designed to treat human immunodeficiency virus (HIV) infection. The CXCR4 receptor is an HIV co-receptor necessary for the virus to penetrate cells. During phase I of clinical studies involving HIV-infected patients and healthy volunteers, a rapid increase in white blood cells after plerixafor administration was observed, accompanied by mobilization of HSCs from the bone marrow into the peripheral blood.[181] Nowadays Plerixafor is approved by the United States Food and Drug Administration (FDA) and the European Medicines Agency (EMA) to induce HSCs mobilization for autologous transplant in patients with non-Hodgkin's lymphoma or multiple myeloma.[182, 183]

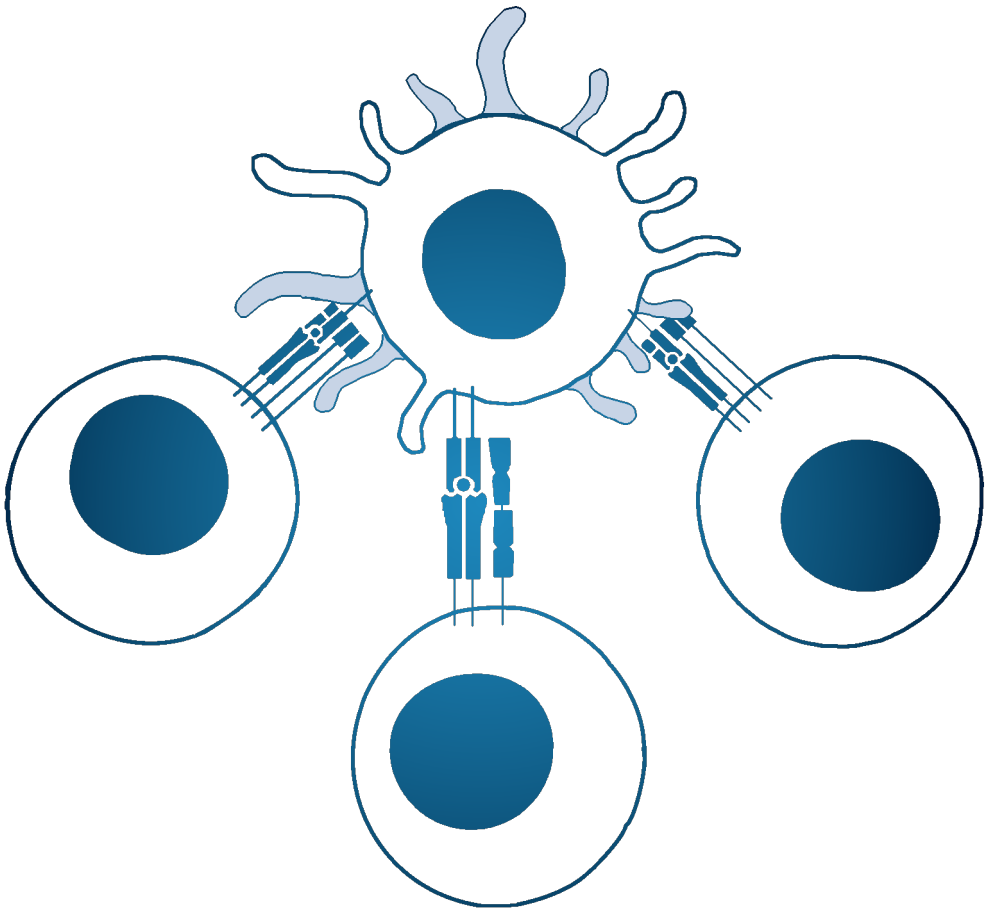
In short, the new autologous HSCT mobilization regimen was started in our IBD Unit in June 2018 and 5 patients were transplanted by May 2019 (Annex Table 3). The complications observed during the mobilization phase in the former protocol (neutropenia, infectious complications and transfusion requirements, among others[131]) have been avoided with the new protocol cohort (Annex Table 4). As expected, the adverse events during the conditioning and transplantation periods were similar to those observed in the previous cohort[131] (Annex Table 5). Regarding

efficacy, 3 of the 4 patients that reached week 26 of follow-up after HSCT achieved clinical remission at that time point, defined as CDAI < 150. Even though more patients and longer follow-up periods need to be included, eliminating cyclophosphamide during mobilization markedly reduces therapy-related morbidity during this phase without apparently affecting treatment efficacy compared to the previous protocol.[130]

The results of this thesis have several implications. First, they show that incomplete immune depletion can lead in to disease remission in some patients, suggesting that less aggressive protocols could equally benefit such patients. Given that safety is the main factor limiting the use of HSCT in larger patient cohorts, this information is highly relevant. While previous studies in other autoimmune diseases have suggested that peripheral immune renewal might explain the induction of remission, we demonstrate here that this change alone does not predict the efficacy of HSCT in Crohn's disease.

Second, our results show a reduction in the intestinal $CD4^+$ memory compartment of patients in remission after HSCT. This observation is unique to the transplantation protocol, since this population did not change to the same extent following anti-TNF treatment. Finally, we have identified a group of Crohn's disease patients with significantly lower peripheral TCR diversity at baseline who failed to achieve the primary endpoint after HSCT. While we do not yet understand the causes underlying this reduced diversity, this observation provides a potential predictor of efficacy, which if confirmed, could be used to better select candidates for HSCT.

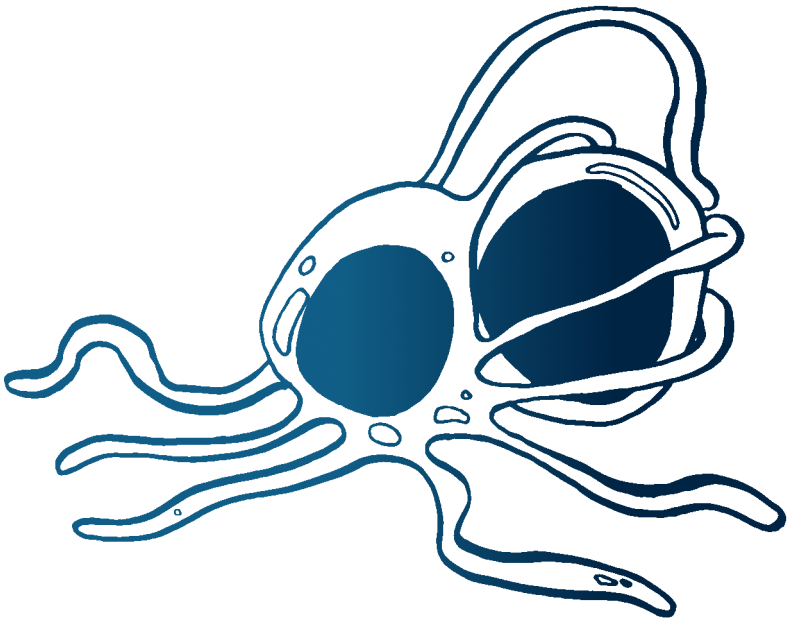
Conclusions



This doctoral thesis, in which we assessed the peripheral and mucosal immune changes that might be involved in inducing remission in Crohn's disease patients undergoing hematopoietic stem cell transplant, allows us to conclude the following:

- HSCT significantly alters the proportions of both T and B lymphocytes in the peripheral and intestinal compartment of Crohn's disease patients.
- The peripheral T cell compartment recovery exhibits different behaviors for $CD4^+$ and $CD8^+$ cells. $CD8^+$ cells recover at 13 weeks after-HSCT. In contrast, naïve $CD4^+$ cells achieve control levels at 1 year post HSCT, while memory $CD4^+$ cells remain decreased at that time-point.
- Naïve B cells are significantly expanded, both in peripheral blood and intestinal mucosa, one year after therapy. Antibody titers against common vaccines are preserved after HSCT, raising the possibility that massive re-immunization might not be required after therapy.
- The mucosal T cell transcriptional signature is significantly reduced in patients who achieve remission following HSCT but not in those undergoing anti-TNF therapy. This observation suggests that mucosal T cell depletion represents an important and unique mechanism of autologous HSCT efficacy in Crohn's disease.
- Due to the conditioning regimen, the number of *TCRB* sequences after HSCT is significantly reduced in blood and biopsy samples. Surviving and new TCR clonotypes expand after HSCT. These changes lead to decreased TCR diversity during the first months following HSCT, which returns to baseline levels one year after treatment.
- Despite extensive remodeling of the lymphocyte compartment, our observation that T cell clones can survive HSCT, both in periphery and mucosa, regardless of efficacy suggests that potentially less aggressive protocols could equally benefit such patients.
- Patients achieving endoscopic remission after autologous HSCT had significantly higher peripheral TCR diversity at baseline. This observation provides a potential predictor of efficacy, which if confirmed, could be used to better select candidates for HSCT.

Acknowledgements



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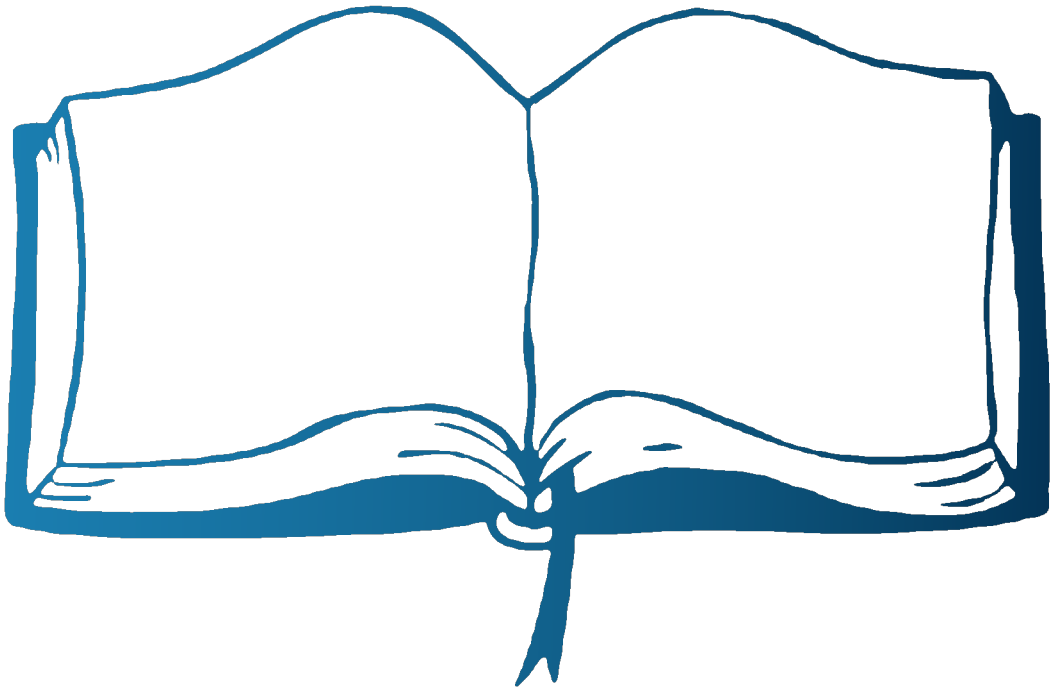

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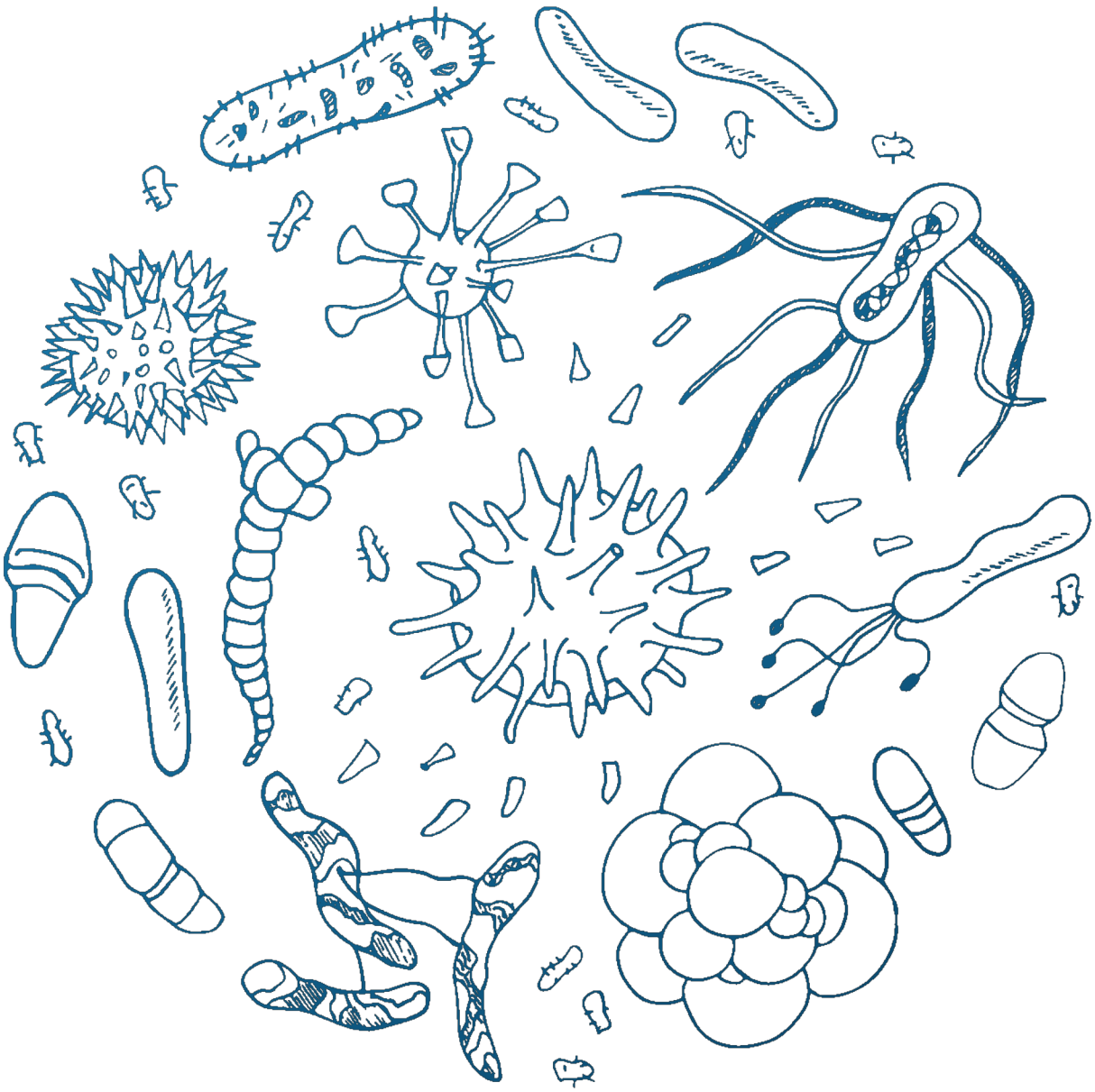
Annex



Annex Table 1. Top 100 genes regulated during remission following HSCT shared by anti-TNF remission signature. Fold changes and FDR values are shown for each gene at week 52 and week 46 by HSCT and anti-TNF, respectively, compared to baseline.

Gene Name	HSCT		Anti-TNF	
	Fold Change	FDR	Fold Change	FDR
CXCL1	-15.56	$1.27x10^{-06}$	-6.51	$3.64x10^{-02}$
FCN1	-13.37	$8.78x10^{-06}$	-6.15	$2.72x10^{-02}$
TCN1	-12.48	$4.03x10^{-17}$	-14.00	$4.38x10^{-02}$
MMP7	-12.12	$3.22x10^{-10}$	-23.59	$2.49x10^{-02}$
TGM2	-11.42	$3.01x10^{-08}$	-3.96	$2.53x10^{-02}$
DUOXA2	-10.35	$2.13x10^{-04}$	-16.57	$4.11x10^{-02}$
CXCL6	-9.96	$1.39x10^{-04}$	-8.89	$3.22x10^{-02}$
IGHG3	-9.86	$6.30x10^{-03}$	-7.16	$1.34x10^{-02}$
ALDH1A2	-9.62	$5.06x10^{-06}$	-12.79	$4.39x10^{-02}$
KCNJ15	-8.75	$1.53x10^{-06}$	-14.44	$3.15x10^{-02}$
TNFRSF6B	-8.56	$8.1x10^{-08}$	-18.38	$2.29x10^{-02}$
IGHG1	-8.45	$8.16x10^{-03}$	-6.96	$2.07x10^{-02}$
SLC6A14	-8.35	$5.5x10^{-07}$	-19.62	$4.04x10^{-02}$
ALPL	-7.94	$1.12x10^{-03}$	-4.46	$2.49x10^{-02}$
HMGCS2	7.79	$8.63x10^{-03}$	4.86	$4.57x10^{-02}$
INHBA	-7.17	$2.17x10^{-04}$	-6.15	$4.57x10^{-02}$
ADAMTS4	-7.13	$8.84x10^{-04}$	-4.01	$2.18x10^{-02}$
NOS2	-6.90	$1.57x10^{-03}$	-4.26	$4.08x10^{-02}$
CHRDL2	-6.85	$2.12x10^{-07}$	-7.66	$4.63x10^{-02}$
CLDN2	-6.58	$1.55x10^{-06}$	-14.13	$4.87x10^{-02}$
SOCS3	-6.58	$2.04x10^{-03}$	-3.46	$3.66x10^{-02}$
DUOX2	-6.21	$1.33x10^{-02}$	-8.05	$2.54x10^{-02}$
TNFRSF10C	-5.96	$3.35x10^{-04}$	-4.26	$3.68x10^{-02}$
HAPLN3	-5.81	$3.55x10^{-05}$	-4.13	$1.88x10^{-02}$
ICAM1	-5.67	$3.87x10^{-04}$	-3.12	$2.83x10^{-02}$
SELP	-5.49	$6.29x10^{-04}$	-2.88	$4.39x10^{-02}$
LAMP3	-5.36	$1.06x10^{-03}$	-3.78	$2.84x10^{-02}$
DYSF	-5.34	$3.53x10^{-03}$	-3.20	$2.49x10^{-02}$
CLEC4E	-5.30	$6.35x10^{-05}$	-9.56	$3.94x10^{-02}$
IL6	-5.24	$4.21x10^{-03}$	-11.71	$4.22x10^{-02}$
FAP	-5.00	$5.86x10^{-04}$	-3.64	$2.89x10^{-02}$
HRH2	-4.92	$2.65x10^{-04}$	-3.26	$3.51x10^{-02}$
FCN3	-4.82	$9.29x10^{-08}$	-7.71	$3.31x10^{-02}$
C2	-4.80	$1.18x10^{-07}$	-2.26	$4.57x10^{-02}$
KRT17	-4.77	$6.72x10^{-07}$	-7.94	$4.17x10^{-02}$
SLC26A2	4.71	$3.39x10^{-03}$	5.69	$3.95x10^{-02}$

Gene Name	HSCT		Anti-TNF	
	Fold Change	FDR	Fold Change	FDR
SOCS1	-4.64	$5.06x10^{-06}$	-4.18	$2.26x10^{-02}$
CHP2	4.41	$1.03x10^{-02}$	3.38	$4.64x10^{-02}$
RAMP3	-4.40	$7.25x10^{-06}$	-2.48	$2.45x10^{-02}$
LCN2	-4.27	$1.99x10^{-02}$	-6.74	$7.13x10^{-03}$
GJA4	-4.26	$2.28x10^{-04}$	-3.05	$3.47x10^{-02}$
CFB	-4.14	$2.69x10^{-06}$	-2.80	$1.92x10^{-02}$
CFP	-4.06	$2.17x10^{-04}$	-3.24	$9.1x10^{-03}$
PTP4A3	-4.05	$3.01x10^{-03}$	-2.87	$1.17x10^{-02}$
IFITM2	-4.02	$6x10^{-05}$	-2.76	$1.05x10^{-02}$
SERPINA3	-4.02	$7.94x10^{-03}$	-14.89	$2.39x10^{-02}$
KIFC3	-3.94	$1.30x10^{-03}$	-2.74	$3.41x10^{-02}$
PCDH17	-3.93	$1.07x10^{-03}$	-3.18	$3.89x10^{-02}$
CX3CL1	-3.92	$6.26x10^{-06}$	-2.17	$2.21x10^{-02}$
GPR4	-3.92	$9.11x10^{-04}$	-3.52	$1.99x10^{-02}$
C2CD4A	-3.92	$6.41x10^{-06}$	-9.26	$1.44x10^{-02}$
CCL22	-3.85	$3x10^{-03}$	-4.26	$2.49x10^{-02}$
TMEM132A	-3.80	$6.16x10^{-03}$	-2.20	$3.02x10^{-02}$
MEOX1	-3.78	$1.07x10^{-03}$	-2.42	$4.87x10^{-02}$
PTGES	-3.77	$5.29x10^{-03}$	-2.16	$4.62x10^{-02}$
PDE4B	-3.73	$2.35x10^{-02}$	-2.85	$4.57x10^{-02}$
ENG	-3.72	$1.79x10^{-03}$	-2.26	$2.84x10^{-02}$
TYMP	-3.69	$2.26x10^{-06}$	-3.42	$5.16x10^{-03}$
FJX1	-3.66	$1.16x10^{-04}$	-3.91	$3.52x10^{-02}$
VASN	-3.65	$5.84x10^{-03}$	-2.68	$3.24x10^{-02}$
TNFRSF4	-3.63	$3.55x10^{-05}$	-4.79	$2.11x10^{-03}$
FOXP3	-3.58	$1.89x10^{-05}$	-2.91	$4.68x10^{-02}$
GNA15	-3.57	$1.51x10^{-05}$	-2.21	$4.28x10^{-02}$
IFITM3	-3.56	$3.63x10^{-11}$	-2.06	$4.96x10^{-02}$
IL4I1	-3.55	$2.84x10^{-03}$	-5.02	$1.17x10^{-02}$
ARHGAP23	-3.53	$1.96x10^{-03}$	-2.29	$4.22x10^{-02}$
FGR	-3.53	$5.15x10^{-03}$	-2.69	$4.45x10^{-02}$
PFKFB3	-3.53	$8.75x10^{-03}$	-2.45	$2.02x10^{-02}$
SOX18	-3.51	$1.47x10^{-04}$	-4.83	$8.54x10^{-03}$
FADS1	-3.50	$6.81x10^{-04}$	-2.17	$4.18x10^{-02}$
APLNR	-3.42	$4.63x10^{-03}$	-2.13	$4.26x10^{-02}$
UGT1A8	3.41	$1.65x10^{-03}$	2.46	$3.4x10^{-02}$
LZTS1	-3.41	$2.84x10^{-04}$	-2.17	$2.63x10^{-02}$
SPHK1	-3.40	$8.69x10^{-03}$	-2.69	$2.09x10^{-02}$
TNFAIP2	-3.36	$3.12x10^{-03}$	-2.07	$4.39x10^{-02}$
PTAFR	-3.33	$2.3x10^{-03}$	-2.27	$4.1x10^{-02}$
FAM167B	-3.30	$6.7x10^{-06}$	-2.47	$4.24x10^{-02}$

Gene Name	HSCT		Anti-TNF	
	Fold Change	FDR	Fold Change	FDR
COL18A1	-3.26	$3.43x10^{-02}$	-3.14	$2.82x10^{-02}$
GPR84	-3.20	$9.67x10^{-03}$	-6.05	$4.04x10^{-02}$
NOS3	-3.18	$6.55x10^{-04}$	-2.61	$2.20x10^{-02}$
PTGDS	-3.20	$8.06x10^{-03}$	-3.70	$4.2x10^{-02}$
RAMP2	-3.19	$2.24x10^{-04}$	-2.19	$3.98x10^{-02}$
MMP9	-3.18	$4.61x10^{-02}$	-3.90	$1.84x10^{-02}$
BATF	-3.18	$9.98x10^{-04}$	-2.88	$2.22x10^{-02}$
PTGIR	-3.17	$3.11x10^{-03}$	-2.79	$4.73x10^{-02}$
SNPH	-3.13	$1.26x10^{-05}$	-2.75	$2.32x10^{-02}$
RASIP1	-3.12	$9.04x10^{-04}$	-2.35	$2.1x10^{-02}$
FSCN1	-3.11	$2.61x10^{-03}$	-3.46	$6.59x10^{-03}$
GGT5	-3.10	$1.35x10^{-02}$	-2.32	$3.47x10^{-02}$
PRKCDBP	-3.10	$2.85x10^{-03}$	-2.85	$3.35x10^{-02}$
VWA1	-3.08	$4.35x10^{-07}$	-2.49	$1.03x10^{-02}$
PDLIM4	-3.07	$8.18x10^{-03}$	-2.14	$4.42x10^{-02}$
MSC	-3.04	$5.61x10^{-03}$	-2.17	$2.83x10^{-02}$
STX11	-3.03	$1.50x10^{-02}$	-3.28	$1.58x10^{-02}$
MADCAM1	-3.02	$4.04x10^{-03}$	-4.43	$1.05x10^{-02}$
MDFI	-3.02	$2.75x10^{-03}$	-5.41	$2.18x10^{-02}$
SHC2	-3.00	$1.25x10^{-05}$	-2.24	$1.41x10^{-02}$
C2CD4B	-2.98	$2.65x10^{-05}$	-7.57	$5.46x10^{-03}$
KIF26B	-2.97	$3.37x10^{-03}$	-2.44	$3.86x10^{-02}$
LY6E	-2.95	$1.25x10^{-03}$	-1.97	$3.77x10^{-02}$

Annex Table 2. Analysis of genes included in the top 10 pathways regulated in the remission-induced HSCT exclusive signature. Fold changes and FDR values are shown for each gene at week 52 and week 46 by HSCT and anti-TNF, respectively, compared to baseline.

Gene Name	HSCT		Anti-TNF	
	Fold Change	FDR	Fold Change	FDR
CXCL5	-19.89	$3.65x10^{-05}$	-6.53	0.24
COL7A1	-6.86	$2.96x10^{-02}$	-2.00	0.19
COL12A1	-5.89	$3.81x10^{-02}$	-1.60	0.51
NOTCH3	-5.81	$9.03x10^{-03}$	-2.14	0.21
FCGR1A	-5.69	$9.95x10^{-05}$	-2.67	0.23
COL4A1	-5.56	$1.98x10^{-02}$	-2.11	0.20
GZMB	-5.40	$3.09x10^{-05}$	-1.93	0.37
FCGR2A	-5.27	$3.46x10^{-03}$	-1.78	0.28
COL15A1	-4.67	$3.29x10^{-02}$	-1.77	0.28
COL8A1	-4.36	$1.41x10^{-02}$	-1.64	0.47
IL2RA	-4.18	$3.58x10^{-04}$	-1.85	0.28
CCL2	-3.96	$7.78x10^{-03}$	-1.39	0.42
IL18RAP	-3.93	$5.89x10^{-04}$	-1.03	0.96
KDR	-3.87	$8.34x10^{-03}$	-1.64	0.34
PDGFRB	-3.86	$2.32x10^{-02}$	-1.58	0.28
EDNRA	-3.32	$1.59x10^{-02}$	-2.31	0.19
FCER1G	-3.28	$2.05x10^{-03}$	-1.43	0.37
FCGR1B	-3.27	$1.07x10^{-04}$	-2.95	0.22
CCR1	-3.20	$1.94x10^{-02}$	-1.46	0.46
PDGFB	-3.05	$1.38x10^{-02}$	-1.40	0.30
IL18R1	-3.03	$1.43x10^{-04}$	-1.23	0.43
FLT1	-3.03	$1.2x10^{-02}$	-1.48	0.39
IGF2	-3.02	$2.02x10^{-02}$	-1.77	0.26
ARHGEF15	-2.99	$2.32x10^{-03}$	-1.62	0.21
HLA-DQA2	-2.85	$3.68x10^{-02}$	-1.28	0.85
PSMB9	-2.82	$6.23x10^{-06}$	-1.57	0.27
RHOJ	-2.82	$7.75x10^{-03}$	-1.33	0.43
AGT	-2.81	$7.01x10^{-03}$	-1.97	0.22
AKT3	-2.76	$1x10^{-02}$	-1.21	0.56
CXCR6	-2.67	$1.18x10^{-03}$	-1.40	0.41
HLA-DQB2	-2.63	$1.02x10^{-02}$	-1.60	0.66
IL1RAP	-2.62	$2.15x10^{-02}$	-1.43	0.21
FGF2	-2.60	$1.12x10^{-02}$	-1.71	0.30
HGF	-2.58	$4.14x10^{-02}$	-1.98	0.29
HLA-DPA1	-2.58	$1.07x10^{-03}$	-1.71	0.20
IFNG	-2.52	$4.86x10^{-04}$	-1.27	0.80

Gene Name	HSCT		Anti-TNF	
	Fold Change	FDR	Fold Change	FDR
TNF	-2.50	$3.44x10^{-02}$	-1.53	0.38
FGFR3	2.49	$9.28x10^{-04}$	1.36	0.34
FYN	-2.47	$6.49x10^{-03}$	-1.50	0.14
CD86	-2.42	$1.15x10^{-02}$	-1.43	0.31
IL2RB	-2.40	$4.28x10^{-03}$	-1.50	0.25
HLA-DPB1	-2.36	$2.52x10^{-03}$	-1.65	0.21
STAT4	-2.35	$2x10^{-03}$	-1.15	0.55
LY96	-2.30	$1.32x10^{-02}$	-1.35	0.42
MYL9	-2.30	$4.23x10^{-02}$	-1.20	0.65
IL10RA	-2.30	$3.43x10^{-02}$	-1.36	0.41
PLA2G4C	-2.29	$3.74x10^{-03}$	-1.25	0.61
GNB4	-2.27	$3.33x10^{-02}$	-1.37	0.33
HAVCR2	-2.27	$1.04x10^{-02}$	-1.43	0.30
CD3D	-2.24	$1.82x10^{-03}$	-1.16	0.68
CD3E	-2.23	$1.84x10^{-03}$	-1.35	0.39
GNG4	2.23	$3.39x10^{-02}$	1.39	0.38
PLCD3	2.22	$1.16x10^{-02}$	1.56	0.24
ADCY4	-2.21	$7.49x10^{-03}$	-1.20	0.28
CCR5	-2.17	$1.89x10^{-02}$	-1.31	0.40
IL12RB2	-2.16	$1.39x10^{-03}$	-1.34	0.61
PLA2G12B	2.15	$1.45x10^{-03}$	2.72	0.30
CD4	-2.15	$1.93x10^{-02}$	-1.40	0.37
ITK	-2.13	$2.29x10^{-02}$	-1.29	0.53
PLCB1	-2.13	$1.67x10^{-02}$	-1.45	0.28
TBX21	-2.11	$1.36x10^{-03}$	-1.33	0.40
PRKCH	-2.10	$6x10^{-03}$	-1.30	0.30
CD28	-2.09	$3.54x10^{-02}$	-1.34	0.50
CCR4	-2.09	$3.13x10^{-02}$	-1.89	0.24
GNG11	-2.09	$1.91x10^{-02}$	-1.41	0.27
CD247	-2.08	$4.7x10^{-03}$	-1.24	0.50
GRAP2	-2.05	$1.71x10^{-03}$	-1.11	0.72
COL8A2	-2.03	$3.46x10^{-02}$	-1.30	0.51
CD40LG	-1.97	$1.85x10^{-02}$	-1.01	0.99
CD14	-1.96	$2.69x10^{-02}$	-1.38	0.22
ARHGEF6	-1.96	$4.71x10^{-02}$	-1.13	0.72
TAP2	-1.95	$4.21x10^{-04}$	-1.38	0.14
TRAT1	-1.92	$9.83x10^{-03}$	-1.23	0.61
ZAP70	-1.92	$4.5x10^{-02}$	-1.39	0.31
CD3G	-1.92	$1.19x10^{-02}$	-1.22	0.57
PRKCQ	-1.88	$4.35x10^{-03}$	-1.08	0.78
FASLG	-1.86	$5.36x10^{-03}$	-1.21	0.56

Gene Name	HSCT		Anti-TNF	
	Fold Change	FDR	Fold Change	FDR
PLEKHA4	-1.86	$2.64x10^{-02}$	-1.56	0.19
GNAO1	-1.85	$3.87x10^{-02}$	-1.01	0.98
NFKBIA	-1.84	$4.33x10^{-02}$	-1.34	0.24
LAT	-1.83	$2.42x10^{-02}$	-1.23	0.47
IFNAR2	-1.82	$4.48x10^{-02}$	-1.36	0.17
JAK2	-1.80	$1.52x10^{-02}$	-1.28	0.28
ADCY3	-1.80	$3x10^{-02}$	-1.20	0.39
APH1B	-1.80	$1.06x10^{-02}$	-1.19	0.26
KL	-1.79	$1.52x10^{-02}$	-1.33	0.38
IGFBP4	-1.79	$2.92x10^{-02}$	-1.10	0.55
NLRC5	-1.78	$2.05x10^{-02}$	-1.20	0.48
IL17RB	1.78	$2.24x10^{-02}$	1.32	0.09
DLL4	-1.77	$9.71x10^{-04}$	-1.09	0.61
ACVR1	-1.75	$3.89x10^{-03}$	-1.27	0.08
HLA-B	-1.75	$8.29x10^{-03}$	-1.32	0.14
KLRD1	-1.75	$2.53x10^{-02}$	1.13	0.72
STAT5A	-1.74	$3.72x10^{-03}$	-1.39	0.06
ARHGEF3	-1.71	$2.38x10^{-02}$	-1.44	0.08
GNB5	-1.71	$1.05x10^{-02}$	-1.36	0.11
TGFBR2	-1.69	$3.62x10^{-02}$	-1.34	0.24
MR1	-1.68	$4.47x10^{-02}$	-1.09	0.68
PRKD1	-1.66	$3.91x10^{-02}$	-1.05	0.88
PLA2G4D	-1.65	$5.52x10^{-03}$	-1.36	0.57
B2M	-1.61	$7.6x10^{-04}$	-1.00	0.99
PLD6	-1.59	$1.9x10^{-02}$	1.02	0.95
PLA2G4E	-1.57	$1.69x10^{-02}$	-1.19	0.78
MYL6B	-1.56	$2.84x10^{-03}$	1.11	0.55
TGFBR1	-1.56	$4.69x10^{-02}$	-1.17	0.27
GNG10	-1.53	$4.48x10^{-02}$	-1.00	0.99
PSMB8	-1.53	$3.72x10^{-03}$	-1.16	0.52

Annex Table 3. Demographic and clinical baseline characteristics of patients included in the new autologous HSCT protocol.

Demographic and clinical baseline characteristics

N	5
Female, no. (%)	4 (80%)
Age at inclusion (years), median (min–max)	38 (20-47)
Smoking habit, no. (%)	3 (60%)
Current smokers	1 (20%)
Disease duration (years), median(min–max)	18 (8-37)
Age at diagnosis (years), no. (%)	
A1 (<16)	2 (40%)
A2 (17–40)	2 (40%)
A3 (>40)	1 (20%)
Location, no. (%)	
L1 (ileal)	3 (60%)
L3 (ileocolonic)	2 (40%)
Behaviour, no. (%)	
B1 (inflammatory)	1 (20%)
B2 (stricturing)	3 (60%)
B3 (penetrating)	1 (20%)
Perianal disease, no. (%)	0 (0%)

Annex Table 4. Complications during the mobilization phase in patients included in the new autologous HSCT protocol.

Complications	**Cyclophosphamide + G-CSF (n=26)**	**G-CSF ± Plerixafor (n=5)**
Hospitalization, days, median (min-max)	18.5 (14-73)	0 (0-0)
Neutropaenia (N<0.5x10^9/L), days, median (min-max)	5 (2-7)	0 (0-0)
Febrile neutropaenia, no. (%)	16 (62%)	0 (0%)
Infectious complication, no. (%)		
Bacteraemia with fever		
Piperacillin-tazobactam-resistant *E. coli* (BC)	1 (4%)	0 (0%)
Bacteraemia with septic shock	2 (8%)	0 (0%)
Labial herpes	1 (4%)	0 (0%)
Non-infectious complications, no. (%)		
Renal failure*	1 (4%)	0 (0%)
Adverse reaction to vancomycin	1 (4%)	0 (0%)
Red blood cell transfusion requirements, no. (%)	13 (50%)	0 (0%)
Arthromyalgia, no. (%)	0 (0%)	1 (20%)

*Sepsis and nephrotoxicity related.
BC, blood culture; CtC, catheter culture.

Annex Table 5. Complications during the conditioning and transplant phases of patients included in the new autologous HSCT protocol.

Complications	**Cyclophosphamide + G-CSF (n=21)**	**G-CSF ± Plerixafor (n=5)**
Hospitalization, days, median (min – max)	26 (24, 43)	26 (23, 35)
Neutrophils low count, days, median (min–max)		
$<0.5\times10^{9}$/L	11 (7, 16)	12 (11, 13)
$<1\times10^{9}$/L	12 (8, 17)	14 (12, 15)
Febrile neutropaenia, no. (%)	20 (95%)	2 (40%)
Fever without septic focus, no. (%)	0 (0%)	2 (40%)
Infectious complication, no. (%)		
Septic shock*	1 (5%)	0 (0%)
Worsening of perianal disease	3 (14%)	0 (0%)
Acute bronchitis	1 (5%)	0 (0%)
Sinusitis	1 (5%)	0 (0%)
Non-infectious complications		
Mucositis I–II (Bearman Scale)	12 (57%)	3 (60%)
Haemorrhagic I–II (Bearman Scale)	2 (10%)	0 (0%)
Drug toxicity		
ATG reaction	6 (29%)	1 (20%)
Mild	3	1
Severe	3	0
Corticoid-related adverse effects		
Adrenal insufficiency	3 (14%)	0 (0%)
Hyperglycaemia	2 (10%)	0 (0%)
Mild psychotic disorder	1 (5%)	0 (0%)
Red blood cell transfusion requirements		
Patients no. (%)	21 (100%)	5 (100%)

* No microorganism was detected.
ATG, antithymocyte globulin; BC, blood culture; CtC, catheter culture; UC, urine culture.

Publications derived from this thesis:

Corraliza AM, Ricart E, López-García A, Masamunt MC, Veny M, Esteller M, Mayorgas A, Le Bourhis L, Allez M, Planell N, Visvanathan S, Baum P, España C, Cabezón-Cabello R, Benítez-Ribas D, Rovira M, Panés J, Salas A. Differences in peripheral and tissue immune cell populations following hematopoietic stem cell transplantation in Crohn's disease patients. *Journal of Crohn's & colitis* Volume 13, Issue 5, May 2019, Pages 634–647

Other publications completed during the candidate's pre-doctoral years (not included in this annex):

Ferrer-Picón E, Dotti I, **Corraliza AM**, Mayorgas A, Esteller M, Perales JC, Ricart E, Masamunt MC, Carrasco A, Tristán E, Esteve M, Salas A. Intestinal inflammation modulates the epithelial response to butyrate in patients with IBD. *Inflammatory Bowel Diseases*, E-pub ahead of print. June 2019

Allez M, Auzolle C, Ngollo M, Bottois H, Chardiny V, **Corraliza AM**, Salas A, Perez K, Stefanescu C, Nancey S, Buisson A, Pariente B, Fumery M, Sokol H, Tréton X, Barnich N, Seksik P, Le Bourhis 2; REMIND Study Group. T cell clonal expansions in ileal Crohn's disease are associated with smoking behaviour and postoperative recurrence. *Gut*, Published Online First: 12 February 2019. doi: 10.1136/gutjnl-2018-317878

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Original Article

Original Article

Differences in Peripheral and Tissue Immune Cell Populations Following Haematopoietic Stem Cell Transplantation in Crohn's Disease Patients

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Abstract

Background and Aims: Recent studies have shown the efficacy of autologous haematopoietic stem cell transplantation [HSCT] in severely refractory Crohn's disease [CD] patients. HSCT is thought to eliminate auto-reactive cells; however, no specific studies of immune reconstitution in CD patients are available.

Methods: We followed a group of CD patients [*n* = 18] receiving autologous HSCT, with 50% of them achieving endoscopic drug-free remission. To elucidate the mechanisms driving efficacy, we monitored changes after HSCT in blood and intestine immune-cell composition. CD patients [*n* = 22] receiving anti-tumour necrosis factor [TNF]-α were included for comparison.

Results: Severe immune ablation followed by HSCT induced dramatic changes in both peripheral blood T and B cells in all patients regardless of the efficacy of the treatment. Endoscopic remission at week 52 following HSCT was associated with significant intestinal transcriptional changes. A comparison of the remission signature with that of anti-TNFα identified both common and unique genes in the HSCT-induced response. Based on deconvolution analysis of intestinal biopsy transcriptome data, we show that response to HSCT, but not to anti-TNFα, is associated with an expansion of naïve B-cells, as seen in blood, and a decrease in the memory resting T-cell content. As expected, endoscopic remission, in response to both HSCT and anti-TNFα, led to a significant reduction in intestinal neutrophil and M1 macrophage content.

Conclusions: Peripheral blood immune remodelling after HSCT does not predict efficacy. In contrast, a profound intestinal T-cell depletion that is maintained long after transplant is associated with mucosal healing following HSCT, but not anti-TNFα.

Key Words: Crohn's disease; autologous haematopoietic stem cell transplantation; anti-TNFα

1. Introduction

Crohn's disease [CD] is a chronic inflammatory disease of the intestinal tract with considerable heterogeneity among affected patients in terms of disease phenotype and therapeutic responses. Despite the increase in the number of drugs approved for the management of CD, a significant percentage of patients remain unresponsive or lose response over time to treatments, and eventually require surgery to control disease activity and/or complications. Nonetheless, in a fraction of these refractory patients, intestinal resection may not be possible due to disease location, extension or previous surgeries. For such patients, autologous haematopoietic stem cell transplantation [HSCT] represents a potential salvage therapy[1] despite the risks associated with this procedure.[2]

Stem cell transplantation is an accepted therapy for haematological disorders, aplastic anaemia and immunodeficiencies. In the context of autoimmune diseases, the serendipitous benefits of transplantation were initially reported in patients suffering from both immune-mediated diseases and haematological disorders. This led to trials that have shown the efficacy of autologous HSCT in treating an array of autoimmune diseases including refractory severe multiple sclerosis [MS],[3,4] systemic lupus erythematosus,[5] juvenile idiopathic arthritis,[6] rheumatoid arthritis[7] and, more recently, CD.[1,8,9]

Indeed, the ASTIC trial recently reported 50% mucosal healing at 1 year after HSCT[9] in a population of patients refractory to all available therapeutic options. In addition, in the largest single-centre cohort study published to date, we showed that HSCT achieves drug-free endoscopic remission in 60% of patients at 1 year of follow-up.[8] Although these data are uncontrolled, they allow the outcome of HSCT to be viewed in the context of reports of novel biological therapies. As an example, recently licensed anti-p40 antibodies [ustekinumab] achieved a response to induction therapy in 34% of patients refractory to anti-tumour necrosis factor [TNF]-α inhibitors; among this subset of initial responders, 53.1% achieved remission at 1 year, which represents just 18% of the whole population enrolled in the study.[10]

The benefit of HSCT in autoimmunity is thought to originate from the ability of intense immune depletion to eliminate autoreactive cells regardless of their specificity. This would lead to *de novo* generation of immune cells that could re-establish tolerance,[11] although no objective evidence of this 'resetting' has been reported thus far. To explore this hypothesis, we monitored a group of 18 CD patients for 1 year after receiving an autologous HSCT. We then compared immune reconstitution both in blood and in intestinal tissue in patients who achieved endoscopic remission and those who did not at that same time point.

2. Material and Methods

Additional information is provided in the Supplementary Methods.

2.1. Patient population and follow-up

Autologous HSCT was considered for CD patients fulfilling the previously described inclusion criteria.[2,8] Given that the mobilization and conditioning protocols are intensely immunosuppressive, additional immunosuppression is avoided as it may potentially pose additional risks during the recovery phase. Anti-TNFα treatment and immunosuppressive drugs were stopped at least 4 and 2 weeks, respectively, before mobilization. The protocol was approved by the Catalan Transplantation Organization and by the local ethics committee. All patients provided written informed consent following extensive counselling. A total of 18 patients were recruited between March 2010 and September 2015. Patient characteristics at inclusion are shown in Table 1. After discharge, patients were closely followed-up.[2,8] In brief, Crohn's Disease Activity Index [CDAI] and laboratory markers were assessed weekly during the first 30 days, and every 6 weeks thereafter. Colonoscopy and/or magnetic resonance imaging were performed at baseline and at weeks 26, 52 and 106 after transplant. The Simple Endoscopic Score for Crohn's Disease [SES-CD] index was used at baseline and during follow-up to assess endoscopic activity. Mucosal healing was defined as SES-CD < 7. Magnetic Resonance Index of Activity [MaRIA] was used at baseline and during follow-up in those patients in whom lesions could not be assessed by ileocolonoscopy. Data are shown in Supplementary Table 1. None of the patients included in this study received any immunosuppressive or biological treatment during the first year of follow-up, with the exception of patient 15 who continued to experience severe lesions 6 months after transplant and started anti-TNFα treatment at that time.

A second cohort comes from an observational prospective study, including CD patients who began treatment with an anti- TNFα antibody [infliximab or adalimumab] and were followed up for 46 weeks. All patients underwent clinical and endoscopic evaluation at weeks 0, 14 and 46. From April 2013 to September 2016, 22 CD patients were included after obtaining written informed consent [Supplementary Table 2]. This study was approved by the Institutional Ethics Committee of the Hospital Clínic de Barcelona [Spain].

Controls [n = 19] were individuals undergoing colonoscopy for mild gastrointestinal symptoms or for colorectal cancer screening, who had a normal examination and no history of inflammatory bowel disease [IBD]. The mean age of this cohort was 53.25 years, ranging from 27 to 69 years; and 10/19 were males.

2.2. Sample collection

Blood samples were collected from patients receiving HSCT at baseline [pre-mobilization] and every 13 weeks after transplant for up to 1 year of follow-up. Blood was collected into PAXgene tubes and frozen at –20°C [PreAnalytiX; Qiagen]. A second blood sample was collected to obtain serum for antibody determination [Supplementary Methods]. An additional 40 mL of blood was used to isolate peripheral blood mononuclear cells [PBMCs]. PBMCs were cryopreserved until later use for cell population analysis.

Colonic and ileal biopsies were collected at the described time points from the involved areas of the intestine of CD patients and from the sigmoid colon or rectum of non-IBD controls. Biopsies were taken at routine colonoscopies, placed in RNAlater RNA Stabilization Reagent [Qiagen] and stored at –80°C until RNA isolation.

2.3. Microarrays

More detailed information on microarrays is given in Supplementary Methods. Transcriptomic analysis of whole blood RNA samples was performed at weeks 0, 13, 26 and 52. RNA was hybridized in Affymetrix chips Human Genome U219. Raw data were analysed using Bioconductor tools in R [v.3.2.3] employing linear models for microarray data [limma v.3.34.1] for differential expression analysis, and adjusting for inter-patient differences [specifying a block argument for patient variable]. Pathway analysis was performed for those genes significantly regulated using Ingenuity Pathways Analysis [IPA, Ingenuity Systems, www.ingenuity.com]. Functional

Table 1. HSCT cohort: patient characteristics at inclusion

	All patients	Remitters	Non-remitters
n	18	9	9
Gender [male/female]	5/13	1/8	4/5
Age [years][a]	29.28 ± 1.76	28.89 ± 1.96	29.67 ± 3.05
Age at diagnosis[b]			
A1 [<16 years]	6 [33]	3 [33]	3 [33]
A2 [17–40 years]	12 [67]	6 [67]	6 [67]
A3 [>40 years]	0 [0]	0 [0]	0 [0]
Disease behaviour[b]			
Inflammatory	13 [72]	7 [78]	6 [67]
Stenosing	1 [6]	1 [11]	0 [0]
Penetrating	4 [22]	1 [11]	3 [33]
Disease location[b]			
L1 [ileal]	0 [0]	0 [0]	0 [0]
L2 [colonic]	4 [22]	2 [22]	2 [22]
L3 [ileocolonic]	9 [50]	4 [45]	5 [56]
L1+L4 [ileal + upper disease]	1 [6]	1 [11]	0 [0]
L3+L4 [ileocolonic + upper disease]	4 [22]	2[22]	2[22]
Disease duration [years][a]	10.33 ± 1.26	9.11 ± 1.66	11.56 ± 1.90
CDAI[a]	268.38 ± 25.13	258.30 ± 23.58	278.46 ± 45.85
SES-CD[a]	22.73 ± 1.90	21.14 ± 2.32	24.13 ± 3.04
Mutated NOD2 [Y/N]	2/16	1/8	1/8

CDAI, Crohn's Disease Activity Index; SES-CD, Simple Endoscopic Score for Crohn's Disease.
[a]Mean ± SEM.
[b]*n* [%].

analysis identified the biological functions that were most significant to the data set.

2.4. RNA sequencing

Barcoded RNA sequencing [RNAseq] libraries were prepared from 500 ng total RNA using Illumina's TruSeq stranded mRNA kit according to the manufacturer's instructions. Libraries were subjected to paired-end sequencing [101 bp] on a HighSeq-4000 platform [Illumina]. Quality filtering was performed using cutadapt v.1.7.1; reads were then mapped against the human reference genome using the STAR aligner v.2.5.2a, and a STAR genome directory was created by supplying the Ensembl gtf annotation file [release GRCh38.10]. Read counts per gene were obtained using the RSEM program v.1.2.31 and the Ensembl gtf annotation file. Following analyses were performed using the R [v.3.2.3] statistical tool. The total number of expressed genes was 24 215. Differential expression analysis was performed with the limma v.3.34.5 and edger v.3.20.6 packages, adjusting for inter-patient differences [specifying a block argument for patient variable]. To correct for multiple testing, the false discovery rate [FDR] was estimated using the method of Benjamini and Hochberg. A gene was considered differentially expressed when it was significant at 5% FDR and showed a fold-change [FC] higher than |1.5|.

2.5. Deconvolution

We used the online analytical platform CIBERSORT v.1.01[12] to estimate the proportions of 22 immune cell types in biopsy samples. Analyses were done with 100 permutations, disabled quantile normalization and default statistical parameters. The results were filtered by a maximum *p*-value of 0.05.

2.6. Statistics

For two time-point comparisons, the Wilcoxon signed-rank test for paired samples was used. For two-group comparisons, the Mann–Whitney–Wilcoxon test was used. Graphs show the mean and standard error of the mean [SEM]. *p*-values ≤0.05 were considered statistically significant.

3. Results

3.1. Whole blood transcriptional analysis reflects changes in leukocyte populations following HSCT

We first analysed the transcriptional signature of peripheral blood by microarray analysis both before mobilization and at immune-ablation [week 0], as well as at different time points [weeks 13, 26 and 52] after HSCT in 14 CD patients from whom blood RNA samples were available [nine remitters]. A total of 199 genes were found to be significantly regulated [186 of them were down-regulated] at week 13 compared to week 0. The majority of these genes [95%] returned to baseline levels by week 52 [Figure 1A]. Interestingly, most of these genes were associated with T-cell functions [IPA analysis, Figure 1B].

A second set of 50 genes [98% of them were up-regulated] showed a delayed modulation, which was significantly regulated at week 26 [but not at week 13] compared to week 0 [Figure 1C]. The majority of these genes [76%] remained up-regulated at week 52. Pathway analysis of this signature revealed the marked-up regulation of B-cell-related functions [Figure 1D]. We compared the transcriptional signatures in the blood of responders and non-responders at all time-points studied, but found no significant differences.

The depletion of T-cell-related genes at week 13 closely correlated with changes in the whole T-cell populations detected in the peripheral blood of 18 CD patients receiving HSCT [Figure 1E]. Further analysis revealed that the decrease in total $CD3^+$ lymphocytes at weeks 13 and 26 was primarily due to the sustained depletion of $CD4^+$ cells at that time [Figure 1E]. In agreement with the expansion of the B-cell transcriptional signature at week 26, we

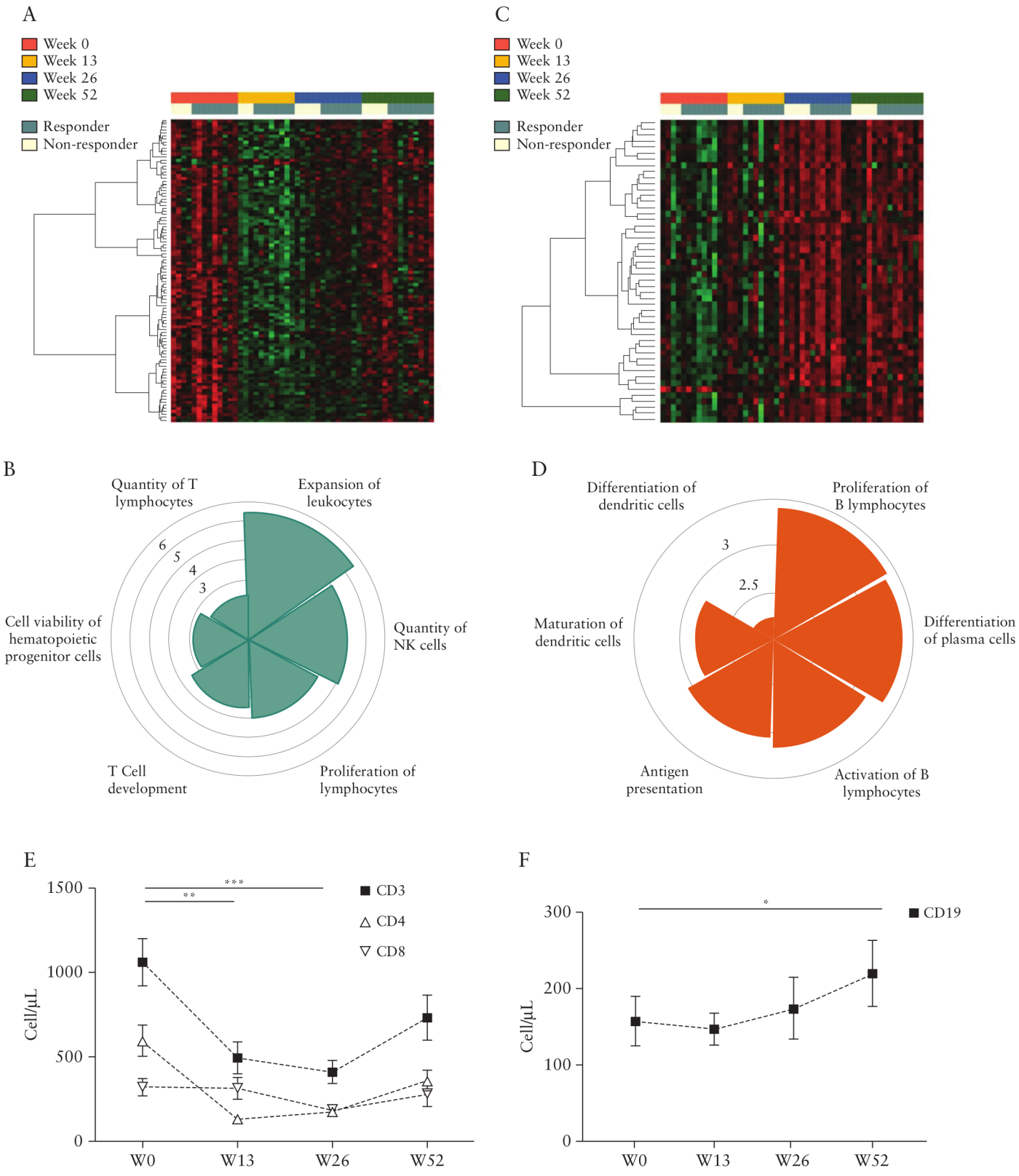

Figure 1. Transcriptional blood signatures following HSCT reveal changes in T and B cells. [A,C] Heatmap representation of microarray expression of top genes regulated at week 13 [A] or week 26 [C] compared to week 0. Each row shows one individual probe and each column an experimental sample. High expression levels are shown in red and low expression levels in green. An unsupervised hierarchical cluster method, using a Pearson distance and average linkage method, was applied for each gene classification. [B,D] Polar graphs showing the top functions identified by ingenuity pathways analysis [IPA] for the genes significantly regulated at week 13 [B] and week 26 [D]. Results are shown graphically as a negative logarithm of the probability score [the most statistically significant pathways have the highest value in the graph]. [E] Absolute numbers (per µL) of blood $CD3^+$, $CD4^+$ and $CD8^+$ cells [mean ± SEM] at baseline [W0] and after haematopoietic stem cell transplantation [HSCT] in Crohn's disease patients [$n = 18$]]. [F] Absolute numbers of blood $CD19^+$ [mean ± SEM] at baseline [W0] and after HSCT in CD patients [$n = 18$]. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; significant by Wilcoxon signed-rank paired test.

observed a trend towards higher B-cell numbers in peripheral blood at week 52 after HSCT compared to baseline [Figure 1F]. However, this increase in total B-cell numbers was not statistically significant. Taken together these results show, as expected, a profound remodelling of the immune cell population after HSCT.

3.2. Naïve and effector/memory T and B cells repopulate the peripheral compartment with different dynamics following HSCT

Within the T helper [$CD3^+CD4^+$] and B [$CD19^+$] cell compartments, we used different cell-surface markers to identify naïve and memory populations by flow cytometry [Figure 2]. At week 13 following transplant, the total number of naïve $CD4^+CD45RA^+$ cells was significantly reduced compared to week 0 [mean reduction of 96%; Figure 2A; $p = 1 \times 10^{-4}$]. $CD45RO^+$ cells were also reduced [mean reduction of 58%; $p = 2 \times 10^{-4}$], although not to the same extent as the $CD45RA^+$ compartment. Despite the marked decrease of naïve $CD4^+$ cells, this subset was recovered by 1 year following HSCT. In contrast, $CD4^+CD45RO^+$ cells remained significantly low at week 52 compared to baseline [$p = 0.029$; Figure 2A], showing that HSCT has a deep and sustained effect on T helper cell populations.

Unlike T cells, at the time points studied, the proportion of $CD27^-$ naïve B cells increased in peripheral blood following HSCT [mean increase of 49%, 70% and 154% at weeks 13, 26 and 52, respectively; Figure 2B]. This increase was statistically significant at 1 year after transplantation [$p = 3 \times 10^{-2}$; Figure 2B], in agreement with the trend shown by transcriptional analysis [Figure 1C and D]. In contrast, activated $CD27^+$ B cells were significantly reduced at weeks 13 and 26 [$p = 7 \times 10^{-3}$ and 0.015, respectively] and fully recovered by week 52 [Figure 2B]. These data suggest that the increase we observed in the transcriptional B-cell signature in blood probably resulted from an expansion in the naïve B-cell compartment following HSCT.

3.3. Changes in peripheral blood populations are not related to the control of CD activity after HSCT

Fifty per cent of our patient cohort achieved endoscopic drug-free remission that was maintained up to 1 year after HSCT [Supplementary Table 1]. As shown in Supplementary Figure 1, both groups of patients showed comparable changes in cell subsets, including $CD3^+$, $CD4^+$, $CD8^+$ and $CD19^+$ subpopulations, following immune ablation and during reconstitution. In agreement with this observation, the following whole blood transcripts underwent significant changes following HSCT that were comparable in all patients regardless of the protocol's efficacy at all time points examined: those expressed by naïve T cells [including recent thymic emigrants], *CCR7* and *PTK7*; *IL7R* and *CD28* genes, both expressed by T cells, and largely by effector memory cells; *CD40LG*, up-regulated by activated T cells; and the B-cell-related genes *CD79A* and *IGHD* [Figure 3]. These data suggest that the lack of efficacy of HSCT may be unrelated to the overall measures of immune ablation used in our study.

3.4. IgG levels in serum remain unchanged after HSCT in CD patients

HSCT impacts the B-cell population in the blood, which may impact antibody production. Hence, we measured changes in serum antibody concentrations following HSCT. Total serum IgA and IgM were significantly decreased in patients following HSCT, while the concentration of total IgG remained unchanged up to 1 year after immune ablation [Figure 4A]. IgG levels against tetanus toxoid [TT] and five other vaccines were measured in serum [Figure 4B and Supplementary Figure 2]. For TT, patients are re-vaccinated about 6 months after transplant, and hence specific antibodies surged at week 52 [Figure 4B]. We also measured IgG anti-*Saccharomyces cerevisiae* antibodies [ASCAs][13] in the same group of patients [Figure 4C]. About 50% of patients presented ASCA levels above the established normal threshold [see Supplementary Methods] at week 0. Changes in ASCAs after transplant varied markedly at the individual level. Remarkably, for those patients with detectable ASCA levels before transplant, a decrease in ASCAs following HSCT did not correlate with disease improvement [Supplementary Figure 2]. We also measured IgG concentrations against *Escherichia coli* flagellin proteins [Fla2, FlaX] and a predicted lipoprotein [YidX], both bacterial proteins previously associated with CD,[14,15] and obtained similar results [Supplementary Figure 2]. These results suggest that while B cells and antibody responses are impacted by HSCT, these parameters are not correlated to treatment efficacy.

3.5. Transcriptional analysis of the intestine reveals differences between the signatures of remission induced by HSCT and anti-TNFα treatments

Biopsies for RNAseq analysis were taken whenever possible from the involved mucosa [colonic and/or ileal] of patients undergoing HSCT at different time points [Supplementary Table 3]. Given that the transcriptomic signatures of colonic and ileal mucosa are markedly different [see Supplementary Figure 3], as well as the fact that the majority of patients had colonic disease [Table 1 and Supplementary Table 3], we limited our analysis to the colonic signatures for the purpose of this study. Differential gene expression analysis was performed between week 0 and different time points [weeks 26 and 52] after HSCT. In remitters [$n = 8$], a total of 1504 protein-coding genes were found to be significantly regulated [1189 of them were down-regulated] at week 26 compared to week 0. At week 52, the number of protein-coding genes significantly regulated from baseline reached 2099 [1730 down-regulated] [Figure 5A]. A common signature containing 1043 genes was significantly regulated at both time points [Figure 5B shows a heatmap representation of the top 100 regulated common genes]. Pathway analysis revealed significant regulation of innate and acquired immune cell activation and recruitment, and cytokine production, amongst others [Figure 5C]. Upstream regulator analysis showed the significant regulation of several key mediators in those patients in remission after HSCT [Figure 5D]. Cytokines such as TNF-α, interferon gamma [IFNγ], interleukin 1 alpha and beta [IL-1α, IL-1β], IL-17A, IL-6, and transforming growth factor beta [TGFβ]; transcriptional factors such as nuclear factor kappa beta [NF-KB], STAT1 and 3, hypoxia-inducible factor-1 [HIF-1] and interferon regulatory factor 1[IRF1]; or enzymes such as prostaglandin synthase 2 [PTFS2] and transglutaminase 2 [TGM2] were all predicted to be upstream regulators of those pathways found to be significantly inhibited 1 year after HSCT.

We next compared the signatures of endoscopic remission obtained following HSCT with those of remission induced by anti-TNFα treatment. To that end, RNAseq analysis of biopsies was performed in a cohort of patients evaluated at baseline and at weeks 14 and 46 following anti-TNFα. Differential gene expression analysis of endoscopic remission at 46 weeks compared to baseline [pre-treatment] showed significant regulation of 1820 genes. In total, 43% of this gene signature [789 genes, Figure 6A, Supplementary Table 4] was also regulated during remission following HSCT. Moreover, 58% of the 2099 genes that changed [FDR < 0.05; FC > 1.5] at

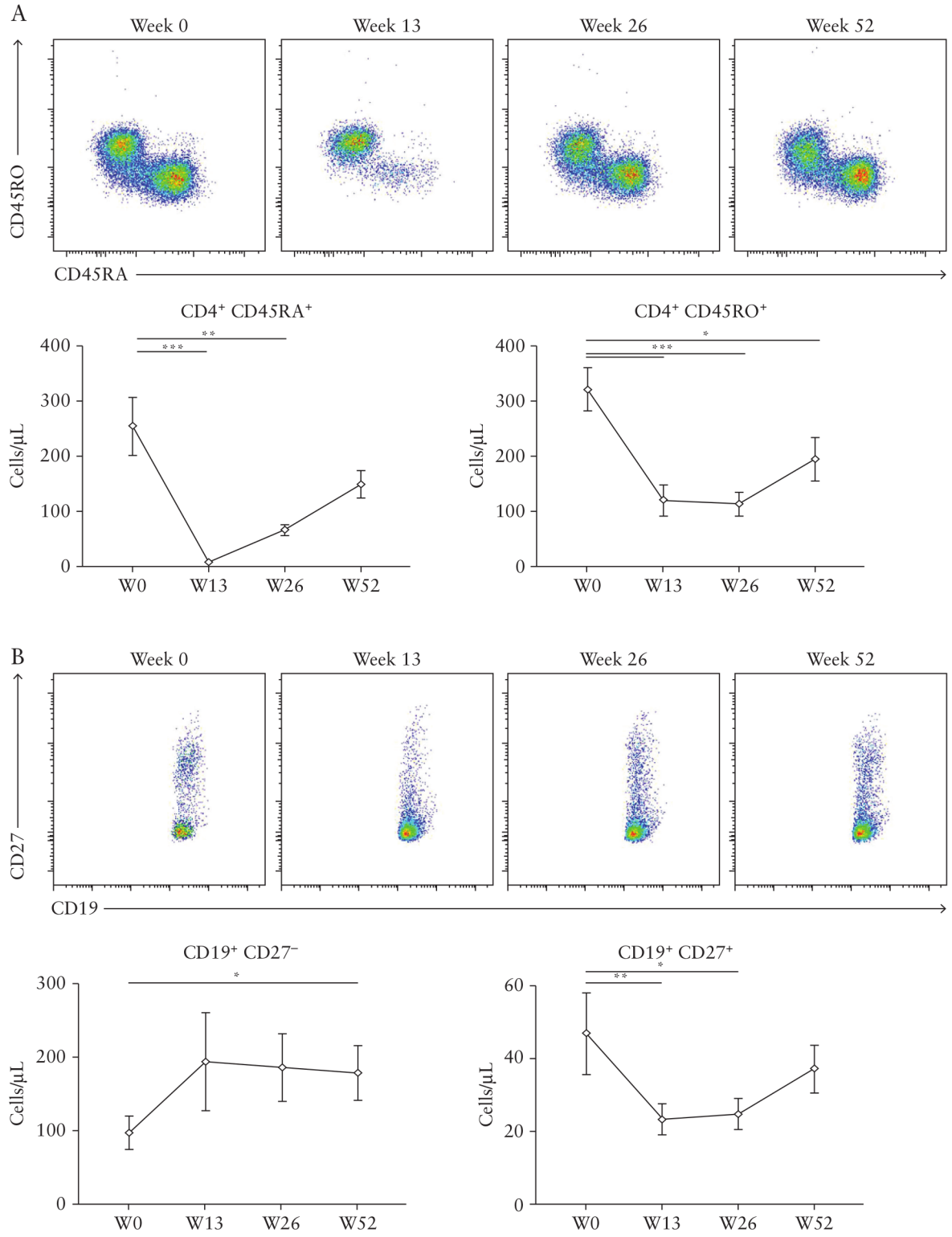

Figure 2. Differential recovery of blood naive and memory cells after autologous haematopoietic stem cell transplantation [HSCT] in Crohn's disease patients. [A] Dot plots representing naïve [CD45RA$^+$] and memory/activated [CD45RO$^+$] compartments within CD4$^+$ T cells at baseline [W0] and after HSCT [W13, W26 and W52]. Data are from one representative patient. Below, the mean ± SEM is represented for all patients included at all time points [n = 18]. [B] Dot plots representing naïve [CD27$^-$] and memory [CD27$^+$] CD19$^+$ B-cell subsets at baseline [W0] and after HSCT [W13, W26 and W52]. Data are from one representative patient. The mean ± SEM for the naïve and memory B-cell absolute numbers is represented [n = 18]. *p < 0.05, **p < 0.01, ***p < 0.001; significant by Wilcoxon signed-rank paired test.

week 52 by HSCT were also regulated, but only when uncorrected by multiple comparisons [nominal p value < 0.05, |FC| > 1.5] in anti-TNF-induced remission [Figure 6A]. Nonetheless, we observed a large number of genes [882] whose expression was exclusively regulated in remitters of the HSCT cohort. Among these HSCT-only regulated signature genes, we observed the significant down-regulation of T-cell-related transcripts [i.e. *CD3E*, *CD28*, *CD3G*, *CD4*]. Indeed, IPA revealed the marked inhibition of canonical pathways

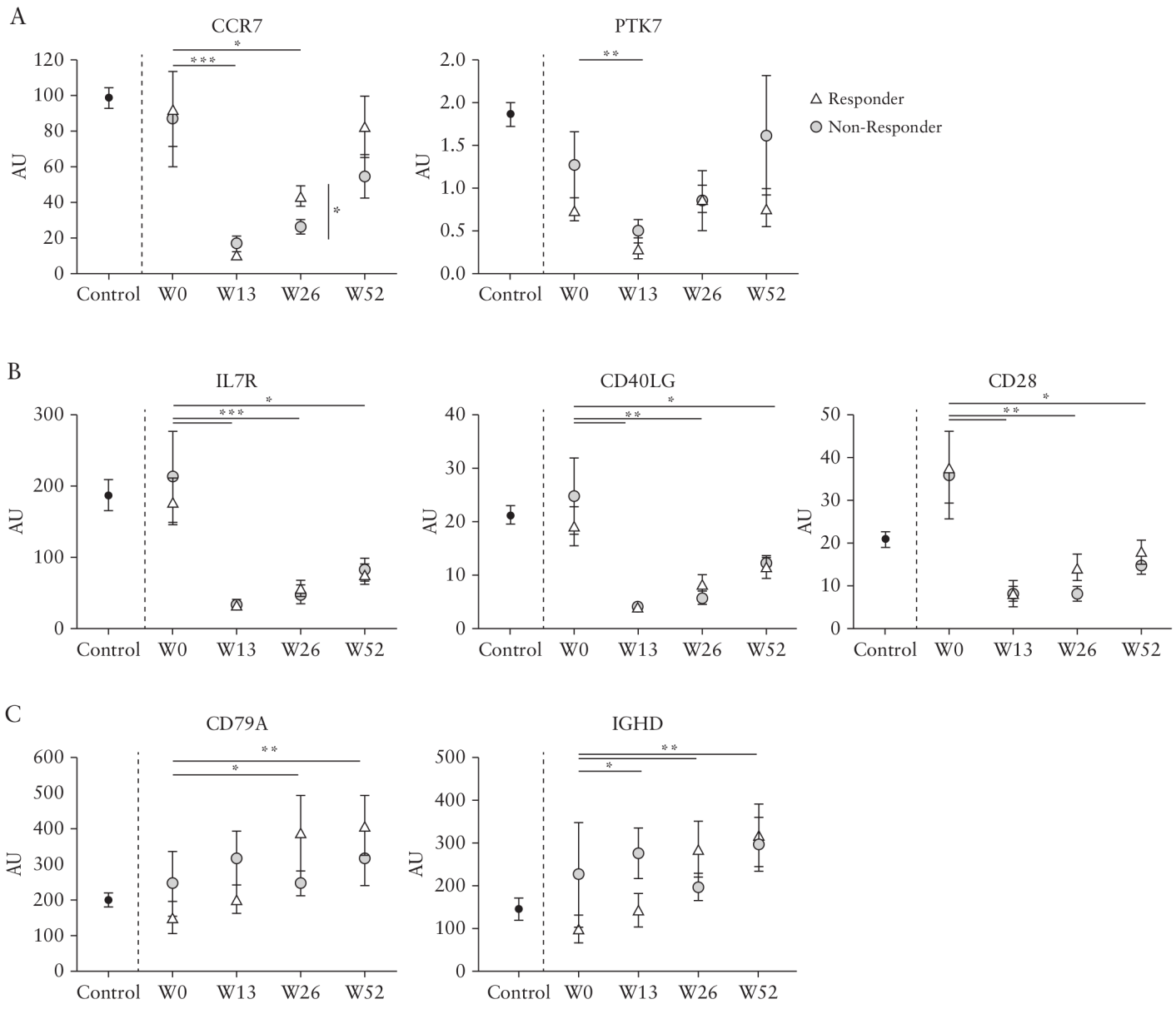

Figure 3. Blood transcriptional analysis of selected T- and B-cell-expressed genes following autologous haematopoietic stem cell transplantation [HSCT]. Relative mRNA expression [mean ± SEM] of CCR7 and PTK7 [A], IL7R, CD40LG, and CD28 [B], and CD79A and IGHD [C] in whole blood of ten healthy non-IBD controls and 18 patients with Crohn's disease [CD] undergoing HSCT at baseline [W0] and after HSCT [W13, W26, W52]. Gene expression in CD patients is shown for remitters and non-remitters separately. Remission to HSCT is defined as drug-free endoscopic remission at W52. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; significant by Wilcoxon signed-rank paired test.

involving T effector functions [Figure 6B, Supplementary Table 5], suggesting that either the number of T cells or their degree of activation was severely diminished in HSCT-induced remission.

Comparison of genes regulated at 1 year of follow-up compared to baseline in anti-TNFα and HSCT shows a very low number of common genes in the non-responder population; only 26 out of the over 1600 regulated [FDR < 0.05] after HSCT were also changed by anti-TNFα [Supplementary Figure 4].

3.6. Immune cell deconvolution analysis reveals unique changes in patients responding to HSCT treatment compared to anti-TNFα

In light of the differences in the transcriptomic remission signatures between anti-TNFα and HSCT treatments, we then analysed changes in the proportions of immune cells in the intestinal mucosa by CIBERSORT deconvolution analysis on the available biopsy RNAseq data. In both cohorts a subset of samples from non-IBD controls were also sequenced side-by-side for comparison.

We observed a significant increase of 'naïve B cells' at 6 months and 1 year following HSCT [Figure 7A] that seemed to closely parallel the expansion observed in peripheral blood [Figure 1]. In addition, patients responding to HSCT exhibited a significant decrease in the 'T cell memory resting' subset [Figure 7B]. None of the anti-TNFα-treated patients, regardless of response, showed comparable changes in these lymphocyte subsets.

Naïve B cells that are expanded up to 1 year following transplant return to pre-transplant levels at 2 years [week 106] of follow-up [Figure 7A]. In contrast, the decrease in the T-cell signature remains constant even up to 2 years after HSCT [Figure 7B]. Hence, deconvolution analyses revealed significant changes in both the B- and the

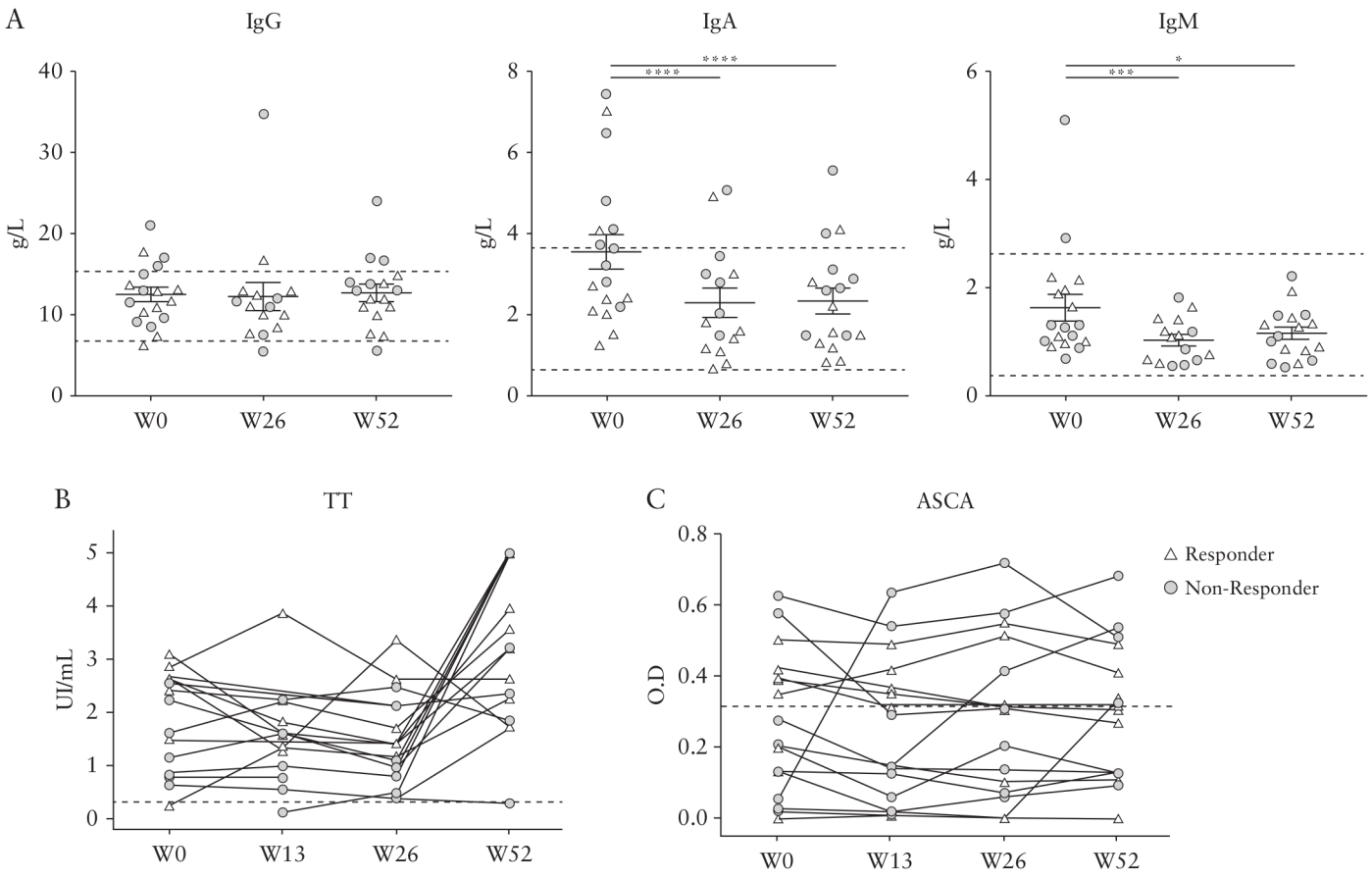

Figure 4. IgG antibody levels in serum remain unchanged after autologous haematopoietic stem cell transplantation [HSCT] in Crohn's disease patients. [A] Serum IgG, IgA and IgM concentrations [g/L; mean ± SEM] at baseline [W0] and after HSCT [W13, W26 and W52] in Crohn's disease patients [$n = 18$]. Dotted line represents the serological protection level. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$; significant by Wilcoxon signed-rank paired test. [B] Anti-tetanus toxoid [TT] antibodies [U.I./mL] detected by ELISA in patient serum at baseline [W0] and after HSCT [W13, W26 and W52]. Dotted line represents the serological protection level. [C] Serum anti-ASCA IgG antibodies at baseline [W0] and after HSCT [W13, W26 and W52]. Values represent extinctions at an optical density [O.D.] at 620 nm obtained by ELISA. The dotted line represents the threshold established for seroreactivity [mean O.D.] in a group of healthy controls.

T-cell mucosal compartments in patients in remission after HSCT, but not in those responding to anti-TNFα.

In contrast, when we looked at innate cells that are abundant in the inflamed mucosa of patients with active CD, we observed a significant decrease of M1 macrophages and neutrophils. This occurred in both the HSCT and the anti-TNFα responding cohorts, correlating with endoscopic remission in both groups of patients [Figure 7C, D]. Taken together, these deconvolution results suggest that changes in both the B- and the T-cell mucosal compartments are not alterations common to a generalized control of inflammation, but are rather unique to HSCT therapy.

To better characterize the changes in the T-cell subsets following HSCT or anti-TNFα, we compared the expression of a selection of T-cell-related [Figure 8A–C] and myeloid-related [Figure 8D] transcripts as measured by RNAseq in both cohorts of patients. While expression of the neutrophil or macrophage markers *CXCL9*, *CXCL10* and *CD16B* was significantly regulated in anti-TNF- and HSCT-responding patients [but not in the non-remitter groups] [Figure 8D], the T-cell markers *CD3E*, *CD28* and *CD40LG*, as well as the T-resident memory marker [T_{RM}] marker *ITGA1* were significantly regulated only in the HSCT-responding cohort. Two other T_{RM} receptors, *CD69* and *ITGAE*, tended to decrease in the HSCT-remitting group, although this was not statistically significant [Figure 8B]. Remarkably, expression of activated effector T cells such as *S1PR1*, *IL2RA* and *CTLA4* were significantly regulated in all patients who achieved an endoscopic response regardless of treatment option [Figure 8C]. Overall, our analysis suggests that endoscopic remission in anti-TNFα- or HSCT-responding patients is associated with a significant decrease in neutrophils, M1 macrophages and activated T-effector cell markers. In contrast, we propose that HSCT has a profound effect on a subset of memory resting and potentially resident T cells that correlates with response to this therapeutic strategy. HSCT seems to markedly decrease total T-cell content in the mucosa of patients who achieve remission.

4. Discussion

The mechanisms that lead to the control of the dysregulated immune response following autologous HSCT are still not entirely clear. The accepted hypothesis is that the conditioning regime eliminates committed pathogenic lymphocyte clones, leading to *de novo* generation of immune cells that re-establish tolerance.[16,17] Indeed, autologous HSCT has been shown to remodel the peripheral immune system, as evidenced by the regeneration of naïve B cells,[11,18] thymic reactivation,[11,19–21] renewal of the T-cell receptor repertoire[11,19,21] and the regeneration of regulatory T cells [Tregs][22,23] in the context of various immune diseases. Nonetheless, there are almost no specific studies that explore immune reconstitution in CD patients following

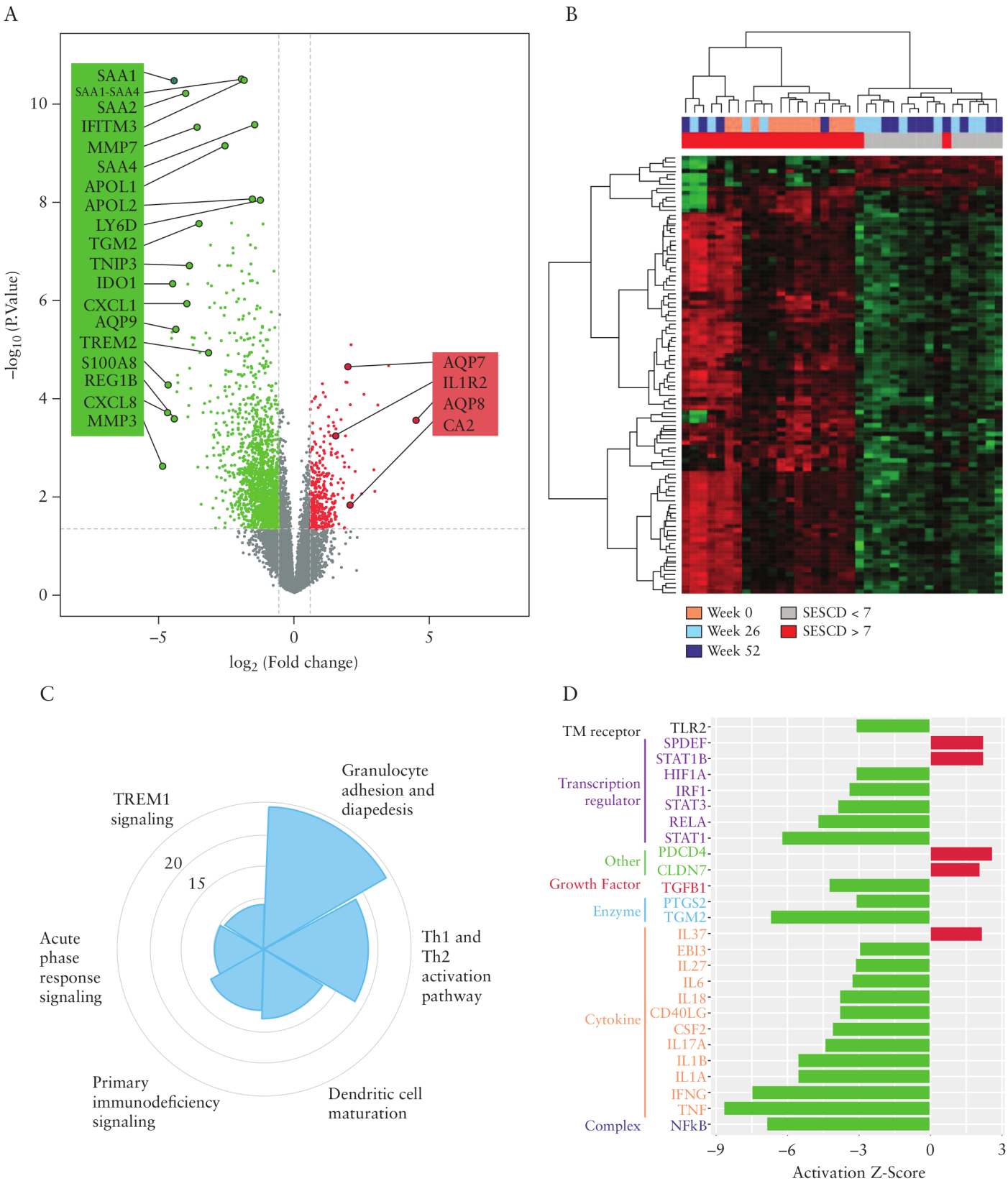

Figure 5. Transcriptional intestinal signatures following HSCT reveal significant changes after HSCT. [A] Volcano plot representation of transcriptional changes in biopsies from patients in remission at week 52 compared to week 0. The $\log_2$[fold change] is shown along the *x*-axis and the $-\log_{10}$[corrected *p*-value] along the *y*-axis. Differentially expressed genes that reach significance [considered as corrected $p < 0.05$ and $|FC|>1.5$] are shown in colour [downregulated genes at week 52 in green and upregulated genes in red]. [B] Heatmap representation of RNAseq expression of the top 100 genes significantly regulated at week 52 [that were also regulated at week 26] compared to week 0. Each row shows one gene and each column an experimental sample. High expression levels are shown in red and low expression levels in green. An unsupervised hierarchical cluster method, using a Pearson distance and average linkage method, was applied for each gene and sample classification. [C] Polar graph showing the top canonical pathways identified by IPA significantly regulated in remission at week 52 compared to week 0. Results are shown graphically as a negative logarithm of the probability score [the most statistically significant pathways have the highest value in the graph]. [D] Bar plot showing the activation z-score of the top upstream regulators at week 52 in patients in endoscopic remission compared to week 0 found by IPA.

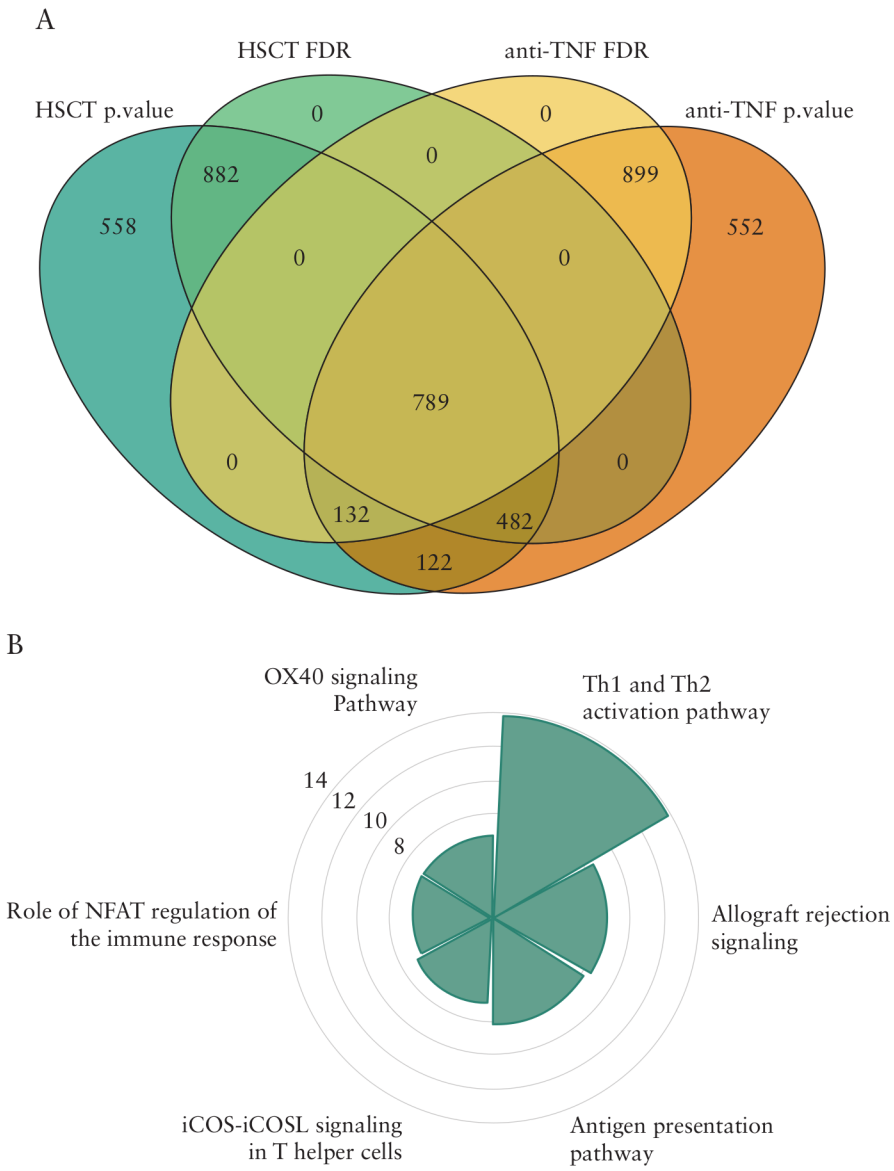

Figure 6. HSCT exclusive remission-induced gene signature is related to down-regulation of T-cell canonical pathways. [A] Venn diagram showing the number of genes that are differentially expressed in CD-remitting colonic mucosa at week 52 after HSCT or week 46 after anti-TNF treatment compared to their respective baseline expression values. Genes are classified based on their significance: FDR < 0.05 or $p < 0.05$. About 40% of the genes [882 transcripts] within the HSCT-induced signature were not changed by anti-TNFα. [B] Polar plot representing the top six pathways significantly regulated in the HSCT-induced signature that is not regulated by anti-TNF. The negative logarithm of the probability score [the most statistically significant pathways have the highest value in the graph] for each pathway is represented.

autologous HSCT. A study from Clerici and collaborators did analyse peripheral immune changes in a cohort of seven CD patients undergoing autologous HSCT.[24] In particular, the authors reported variations in the percentage of peripheral blood monocytes that produced TNFα and IL-10 at 6 and 12 months after autologous HSCT. In a subset of three of four patients defined as 'full responders' [patients who achieved endoscopic remission 6 months after transplant], they observed a transient increase in the percentage of circulating Treg cells during the first 6 months. Except for this limited characterization, no study to date has monitored the immune changes that take place in peripheral blood of CD patients receiving autologous HSCT. Furthermore, to our knowledge no data are available on the cellular and molecular changes that take place in the mucosa of these patients.

Our data clearly show that 3 months after severe immune ablation and HSCT, the peripheral blood CD4⁺ T-cell compartment remains compromised, while the CD8⁺ T- and the B-cell subsets have completely recovered. Moreover, and in agreement with the existing literature,[25] naïve CD4⁺ T cells remain almost completely depleted at the 3-month follow-up time point, but then recover to baseline levels by 1 year after transplant. This supports the notion that CD patients reconstitute the naïve T-cell compartment through mechanisms of homeostatic proliferation and/or thymic output. In contrast to the naïve compartment, circulating memory T cells, while significantly reduced compared to baseline at week 13, are not completely depleted at that time, suggesting that memory clones may survive chemotherapy or may be reintroduced in the graft. Despite this partial apparent persistence, the CD4⁺ memory compartment showed a delayed recovery, in agreement with the existing literature.[26] On the other hand, CD8⁺ cells rely mostly on the peripheral expansion of mature cells, and not on thymic function, and as we confirm here are rapidly replenished after transplant.[26,27] This disconnect between

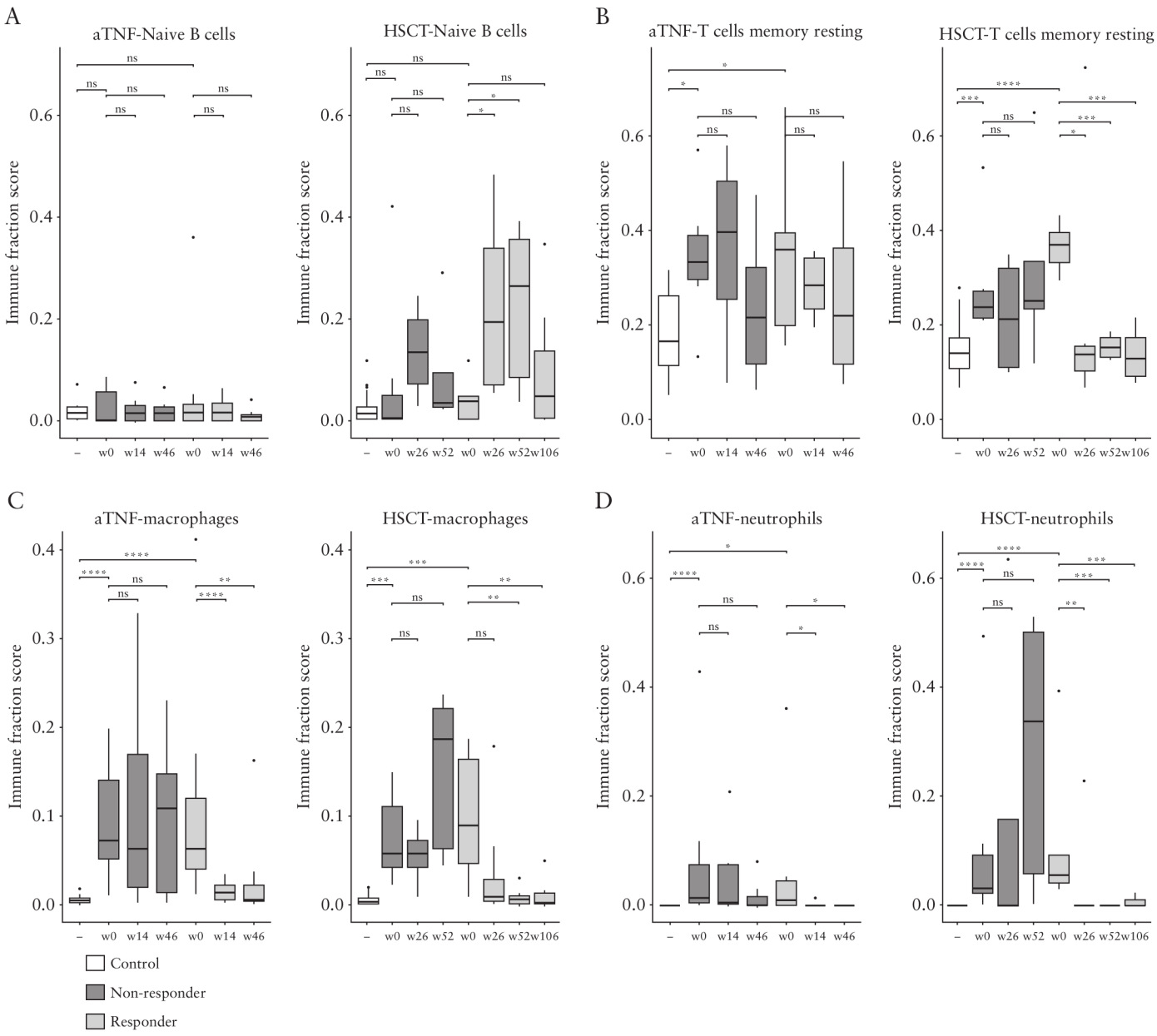

Figure 7. Immune cell deconvolution analysis reveals unique changes in intestinal cellular composition in patients responding to HSCT treatment compared to anti-TNFα. [A] Cell deconvolution analysis [CIBERSORT] of biopsy samples from healthy non-IBD controls [n = 19] and CD patients before and after treatment with anti-TNFα [n = 22, 13 responders] and HSCT [n = 14, 8 responders]. The figure shows the results for naïve B cells [A], resting memory T cells [B], M1 macrophages [C] and neutrophils [D]. Information on colon biopsy samples from responders to HSCT at week 106 [2 years following HSCT; n = 7] is also included in this analysis. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; significant by Mann–Whitney–Wilcoxon test.

replenishment of the CD4 and CD8 compartments after HSCT has also been observed in MS patients.[28]

The rapid recovery and later expansion of the B-cell compartment we observed after HSCT was also in agreement with previous findings[11]; however, the persistence of IgG antibody titres was unexpected. Previous studies have shown a decrease in circulating immunoglobulins following high-dose chemotherapy and autologous $CD34^+$ reconstitution,[29] with serum IgM and IgA levels still significantly decreased 9 months after reconstitution. Indeed, we observed a significant decrease in serum IgA and IgM immunoglobulins at 6 and 12 months of follow-up. This reduction, however, was also detected in non-remitters. On the other hand, IgG levels remained unchanged regardless of the transplant's efficacy, and protection towards common vaccines based on antibody serum levels was not lost after HSCT, in contrast to what has been previously reported.[11] It is important to note that much of the evidence on antibody titres after transplant is based on patients subject to more intense conditioning regimes,[29] raising the possibility that the less aggressive protocol used for immune-mediated diseases does not require massive re-immunization afterwards. Nevertheless, this needs to be confirmed in a larger patient cohort.

Therefore, our study shows that monitoring the depletion and recovery of the peripheral immune compartment after HSCT provides a measure of the extent of the immune depletion, but does not correlate with the response [endoscopic remission] to the HSCT therapy.

Hence, we focused our analysis on the intestinal tissue in areas of active inflammation at baseline, and compared the transcriptional signatures of those segments before and after HSCT. To our

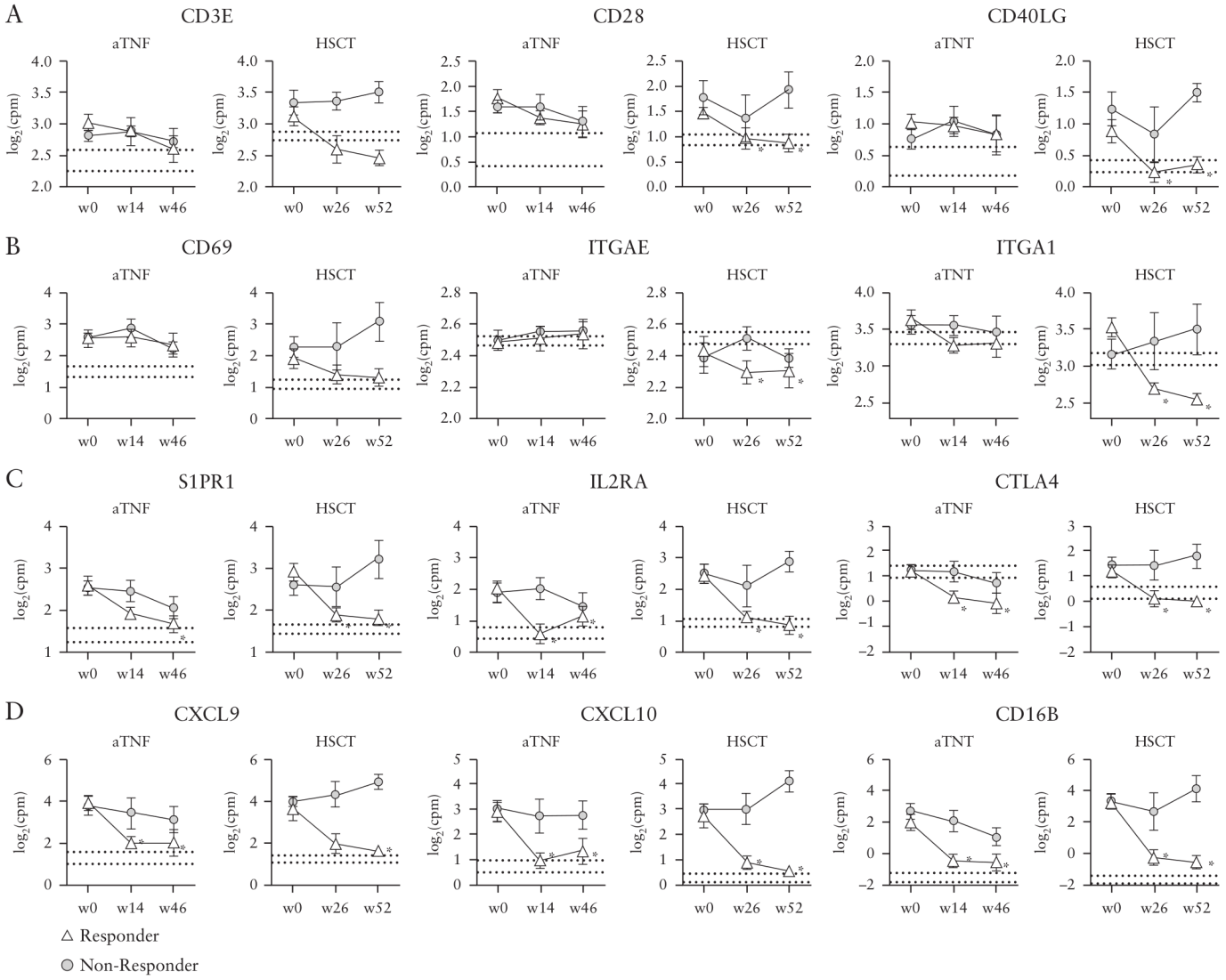

Figure 8. T-cell-related gene expression by RNAseq before and after treatment with anti-TNFα and HSCT. Gene expression was determined in intestinal biopsies by RNAseq in CD patients treated with anti-TNFα therapy or autologous HSCT. The expression values are normalized and represent log-transformed counts for each gene. Dotted lines show the SEM for each gene from the control samples in each cohort. Expression at different time points for endoscopic remitters and non-remitters is shown for T-cell-related genes *CD3E*, *CD28* and *CD40LG* [A]; tissue-resident T cells [T_{RM}] *CD69*, *ITGEA* and *ITGA1* [B], activated effector T cells *S1PR1*, *IL2RA* and *CTLA4* [C], and neutrophils or the macrophage genes *CXCL9*, *CXCL10* and *CD16B* [B]. Asterisks mark the statistically significant differences in each group for each treatment compared to baseline, by Mann–Whitney–Wilcoxon test; *$p > 0.05$.

knowledge, this is the first in-depth characterization of the cellular and molecular mechanisms leading to remission induced by autologous HSCT. Transcriptional analysis of the biopsies revealed over 2000 genes being significantly regulated in remitting patients. This signature comprised hundreds of genes that are also markedly regulated by anti-TNF therapy and included cytokine-mediated inflammatory pathways, genes expressed by infiltrating immune cells such as granulocytes and activated macrophages, as well as tissue remodelling and regenerating pathways that have been extensively described to be modulated in response to conventional treatment.[30,31] This analysis clearly shows that in this group of CD patients [50% of our study cohort], HSCT was able to drive mucosal healing associated with profound molecular changes characteristic of disease remission.

Remarkably, about 50% of the signature of HSCT-remitters was not shared by anti-TNFα remitters. This HSCT-exclusive signature primarily comprised T-cell-related genes, suggesting changes in the mucosal T-cell content and phenotype following HSCT. This observation was further confirmed by deconvolution analysis. This computational approach looks at whole genome transcriptional signatures from complex tissues such as the intestinal lamina propria to enumerate cell subsets present at different time points. Using this approach, we characterized and inferred changes in tissue cell composition following HSCT. While achieving endoscopic remission in response to HSCT or anti-TNFα therapy was associated with changes in the neutrophil and M1 macrophage proportions in biopsies, changes in other immune cellular subsets, such as naïve B cells or certain populations of T cells, were only observed after transplant.

The unique expansion of naïve B cells within the mucosa of patients in remission after HSCT closely correlated with the changes observed in peripheral blood in these patients, and as mentioned, was not observed in response to anti-TNFα treatment. The significance of this B-cell expansion is difficult to ascertain. However, based on preliminary data on both blood and tissue samples at 2 years after transplant, this expansion is temporary and may merely reflect

the enlargement of this compartment following the severe aplasia induced by the conditioning protocol.

Potentially more relevant to the mechanism of action at work in the transplantation protocol is the significant decrease in the intestinal $CD4^+$ memory compartment of patients in remission after HSCT. This population did not change to the same extent from anti-TNFα treatment, revealing a mechanism unique to the transplantation protocol. Anti-TNFα has been shown to partially modulate the mucosal T-cell repertoire, acting primarily on highly expanded clones.[32] In fact, in our cohort we did see a significant down-regulation of certain T-cell-related genes, especially those related to the activated effector compartment. Indeed, induction of T-cell apoptosis has been described to take place as rapidly as 24 h after anti-TNFα treatment.[33,34] While anti-TNFα can therefore act on the T-cell infiltrate, the study by Doorenspleet *et al.* showed that a considerable part of the repertoire in the mucosa of CD patients persisted regardless of the response to this biological.[32]

Our data suggest that HSCT achieved a significant reduction in total T-cell mucosa content, as seen by the significant down-regulation of genes such as *CD3E* and *CD28*. This suggests that transplant has a profound effect on the overall T-cell content that is unique to the stem cell transplant therapy and is probably induced by the ablative treatment. This effect, in contrast to the depletion of T cells in the peripheral blood, was not seen in non-responders to HSCT, suggesting strongly that depletion of T cells in the mucosa is linked to the efficacy of this treatment. One possibility that would need further study is that HSCT, but not anti-TNFα, can act on the tissue-resident T-cell compartment [T_{RM}], which constitutes a predominant T-cell subset in healthy mucosal tissues.[35] These non-migratory T cells are poorly characterized in the context of CD and have been described as acquiring *CD69* expression [while losing *S1PR1* upon tissue entering], as well as the integrins αE [*CD103*] and α1 [*ITGA1*], which only decrease in the HSCT-remitting cohort.

In summary, our study provides the first in-depth description of immune cell depletion and reconstitution following immune ablation and HSCT in a group of CD patients. While previous studies in other autoimmune diseases have suggested that peripheral immune renewal might explain the induction of remission, we demonstrate here that this change alone does not predict efficacy of HSCT in CD. In contrast, we show that remission induced by HSCT, but not by anti-TNFα therapy, results in a significant decrease in the mucosal T-cell content, suggesting that this represents an important mechanism of HSCT efficacy in CD.

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Conflict of Interest

SV and PB are Boehringer Ingelheim Pharmaceuticals Inc. employees. AS has received consultancy fees and grant money from Boehringer Ingelheim Pharmaceuticals Inc. JP has received grant money from MSD.

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Author Contributions

AS, ER and JP designed the experiments. AMC and ME performed most of the experiments. CE, RC-C and DB-R designed and performed the flow cytometry experiments. AM, AL, MR, MCM and ER recruited patients and collected patient data and samples. SV and PB performed the RNAseq of the anti-TNFα cohort data. NP collected and analysed anti-TNFα cohort data. AS and AMC analysed all of the data. AMC, AS and MV wrote the manuscript with contributions from JP, ER, MR, LLB and MA. All authors reviewed the manuscript before submission.

Transcript Profiling

Blood microarray raw data and biopsy RNAseq data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE100922 for blood and GSE115390 for biopsy data.

Supplementary Data

Supplementary data to this article can be found online at ECCO-JCC online.

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