



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]- $\alpha$  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-TNF $\alpha$  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-TNF $\alpha$ , 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-TNF $\alpha$ , 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-TNF $\alpha$ .

**Key Words:** Crohn's disease; autologous haematopoietic stem cell transplantation; anti-TNF $\alpha$

## 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<sup>1</sup> despite the risks associated with this procedure.<sup>2</sup>

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],<sup>3,4</sup> systemic lupus erythematosus,<sup>5</sup> juvenile idiopathic arthritis,<sup>6</sup> rheumatoid arthritis<sup>7</sup> and, more recently, CD.<sup>1,8,9</sup>

Indeed, the ASTIC trial recently reported 50% mucosal healing at 1 year after HSCT<sup>9</sup> 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.<sup>8</sup> 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]- $\alpha$  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.<sup>10</sup>

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,<sup>11</sup> 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.<sup>2,8</sup> 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-TNF $\alpha$  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.<sup>2,8</sup> 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-TNF $\alpha$  treatment at that time.

A second cohort comes from an observational prospective study, including CD patients who began treatment with an anti-TNF $\alpha$  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  $-20^{\circ}\text{C}$  [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  $-80^{\circ}\text{C}$  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](http://www.ingenuity.com)]. Functional

**Table 1.** HSCT cohort: patient characteristics at inclusion

	All patients	Remitters	Non-remitters
<i>n</i>	18	9	9
Gender [male/female]	5/13	1/8	4/5
Age [years] <sup>a</sup>	29.28 ± 1.76	28.89 ± 1.96	29.67 ± 3.05
Age at diagnosis <sup>b</sup>			
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 <sup>b</sup>			
Inflammatory	13 [72]	7 [78]	6 [67]
Stenosing	1 [6]	1 [11]	0 [0]
Penetrating	4 [22]	1 [11]	3 [33]
Disease location <sup>b</sup>			
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] <sup>a</sup>	10.33 ± 1.26	9.11 ± 1.66	11.56 ± 1.90
CDAI <sup>a</sup>	268.38 ± 25.13	258.30 ± 23.58	278.46 ± 45.85
SES-CD <sup>a</sup>	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.

<sup>a</sup>Mean ± SEM.

<sup>b</sup>*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<sup>12</sup> 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.

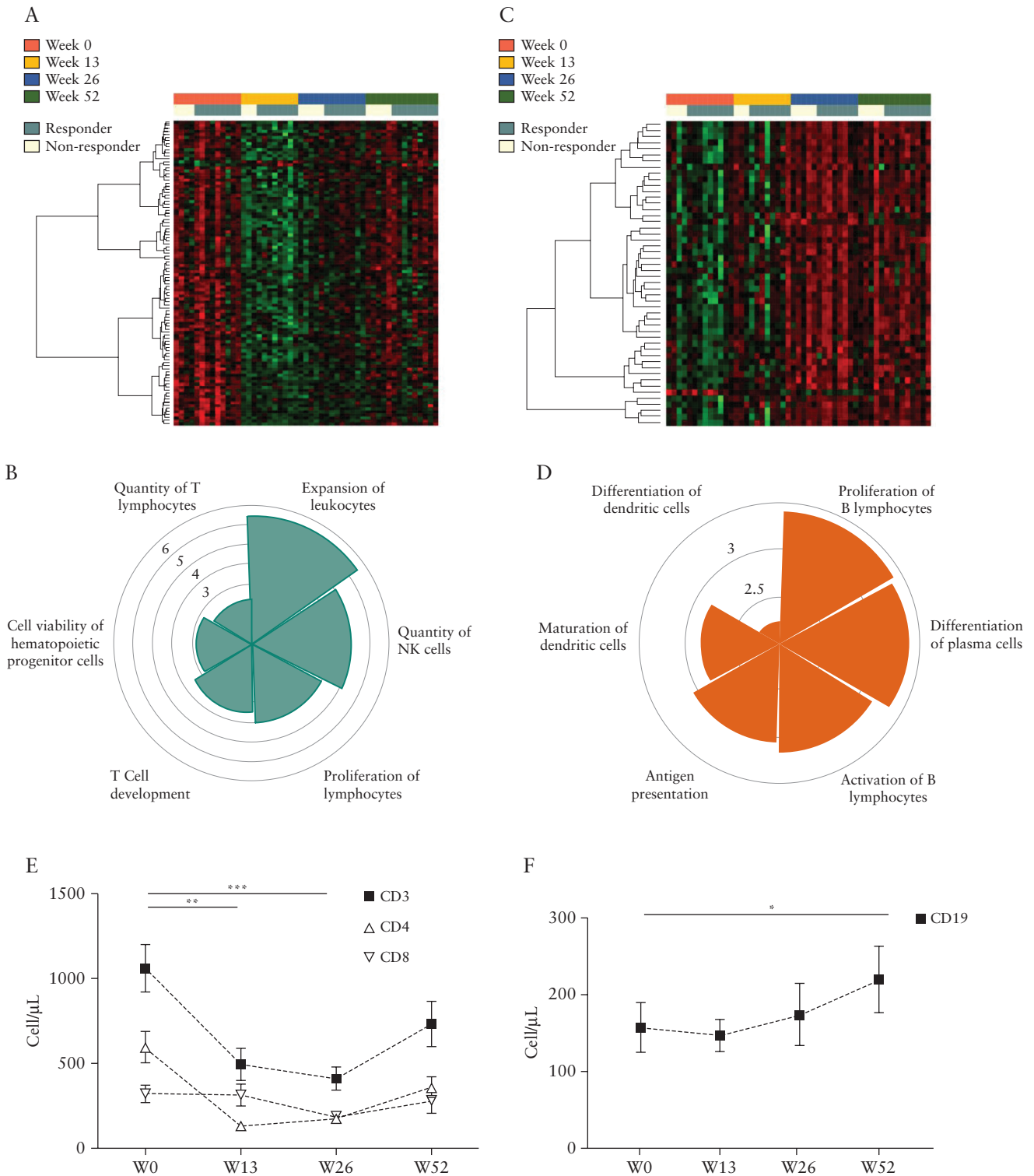
### 3. Results

#### 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<sup>+</sup> lymphocytes at weeks 13 and 26 was primarily due to the sustained depletion of CD4<sup>+</sup> 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  $\mu\text{L}$ ) of blood CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> cells [mean  $\pm$  SEM] at baseline [W0] and after haematopoietic stem cell transplantation [HSCT] in Crohn's disease patients [ $n = 18$ ]. [F] Absolute numbers of blood CD19<sup>+</sup> [mean  $\pm$  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 remodeling 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<sup>+</sup>CD4<sup>+</sup>] and B [CD19<sup>+</sup>] 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<sup>+</sup>CD45RA<sup>+</sup> cells was significantly reduced compared to week 0 [mean reduction of 96%; Figure 2A;  $p = 1 \times 10^{-4}$ ]. CD45RO<sup>+</sup> cells were also reduced [mean reduction of 58%;  $p = 2 \times 10^{-4}$ ], although not to the same extent as the CD45RA<sup>+</sup> compartment. Despite the marked decrease of naïve CD4<sup>+</sup> cells, this subset was recovered by 1 year following HSCT. In contrast, CD4<sup>+</sup>CD45RO<sup>+</sup> 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<sup>-</sup> 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<sup>+</sup> 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<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup> and CD19<sup>+</sup> 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

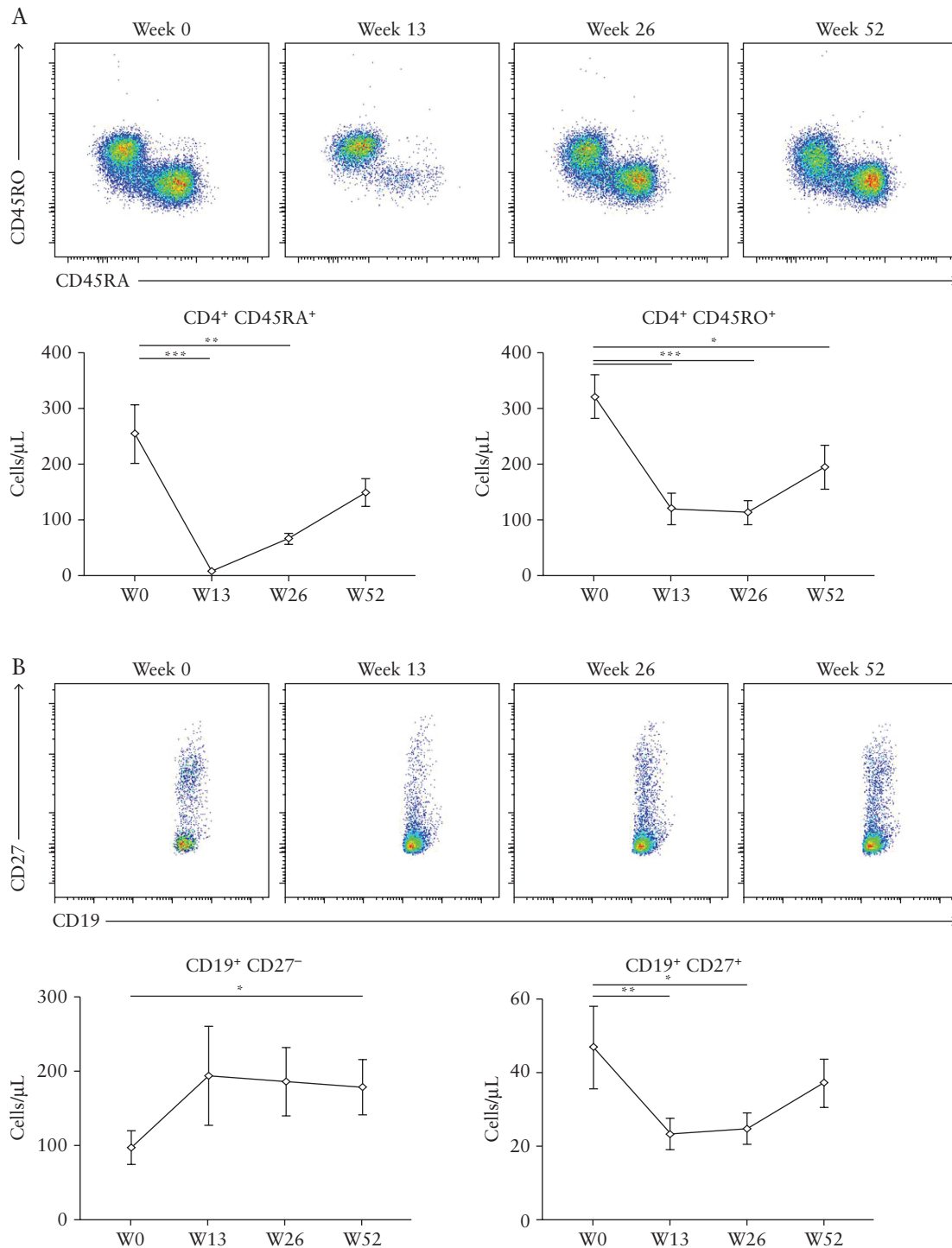
HSCT impacts the B-cell population in the blood, which may impact antibody production. Hence, we measured changes in serum antibody concentrations following HSCT. Total serum IgA and IgM were significantly decreased in patients following HSCT, while the concentration of total IgG remained unchanged up 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]<sup>13</sup> 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,<sup>14,15</sup> 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-TNF $\alpha$ 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- $\alpha$ , interferon gamma [IFN $\gamma$ ], interleukin 1 alpha and beta [IL-1 $\alpha$ , IL-1 $\beta$ ], IL-17A, IL-6, and transforming growth factor beta [TGF $\beta$ ]; 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.

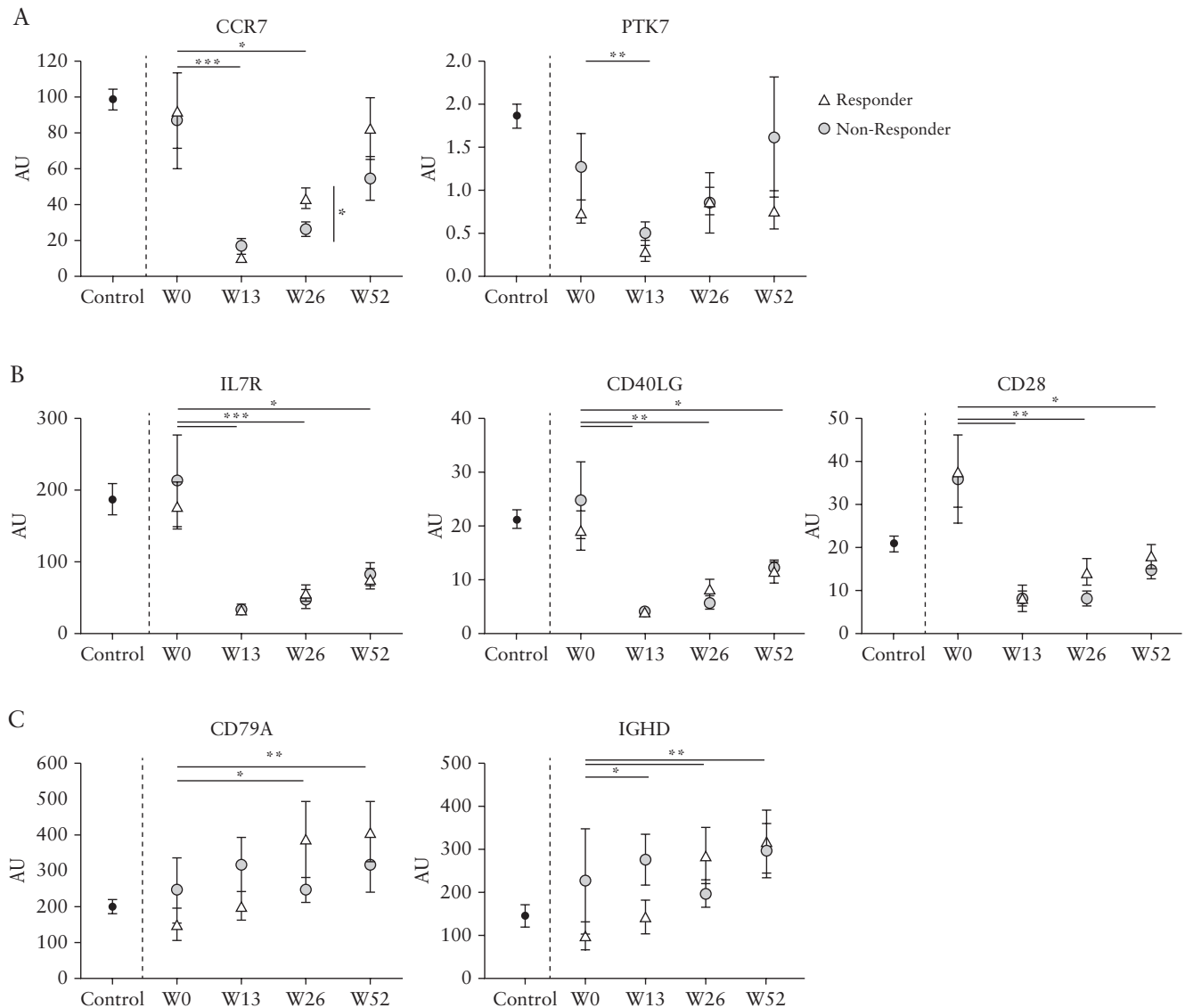
We next compared the signatures of endoscopic remission obtained following HSCT with those of remission induced by anti-TNF $\alpha$  treatment. To that end, RNAseq analysis of biopsies was performed in a cohort of patients evaluated at baseline and at weeks 14 and 46 following anti-TNF $\alpha$ . Differential 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 naïve and memory cells after autologous haematopoietic stem cell transplantation [HSCT] in Crohn's disease patients. [A] Dot plots representing naïve [CD45RA<sup>+</sup>] and memory/activated [CD45RO<sup>+</sup>] compartments within CD4<sup>+</sup> 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<sup>-</sup>] and memory [CD27<sup>+</sup>] CD19<sup>+</sup> 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  $\pm$  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-TNF $\alpha$  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-TNF $\alpha$  [Supplementary Figure 4].

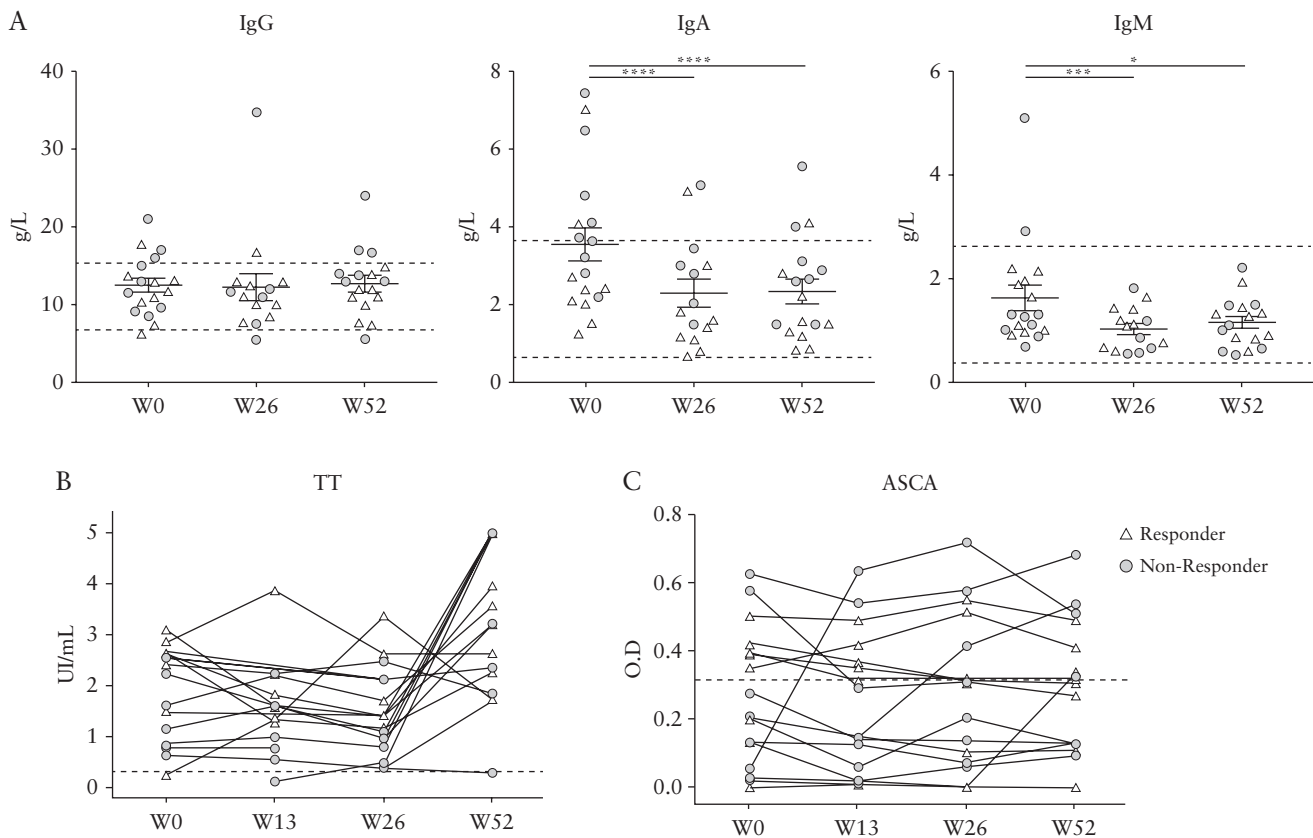
### 3.6. Immune cell deconvolution analysis reveals unique changes in patients responding to HSCT treatment compared to anti-TNF $\alpha$

In light of the differences in the transcriptomic remission signatures between anti-TNF $\alpha$  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-TNF $\alpha$ -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  $\pm$  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-TNF $\alpha$ .

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-TNF $\alpha$  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.

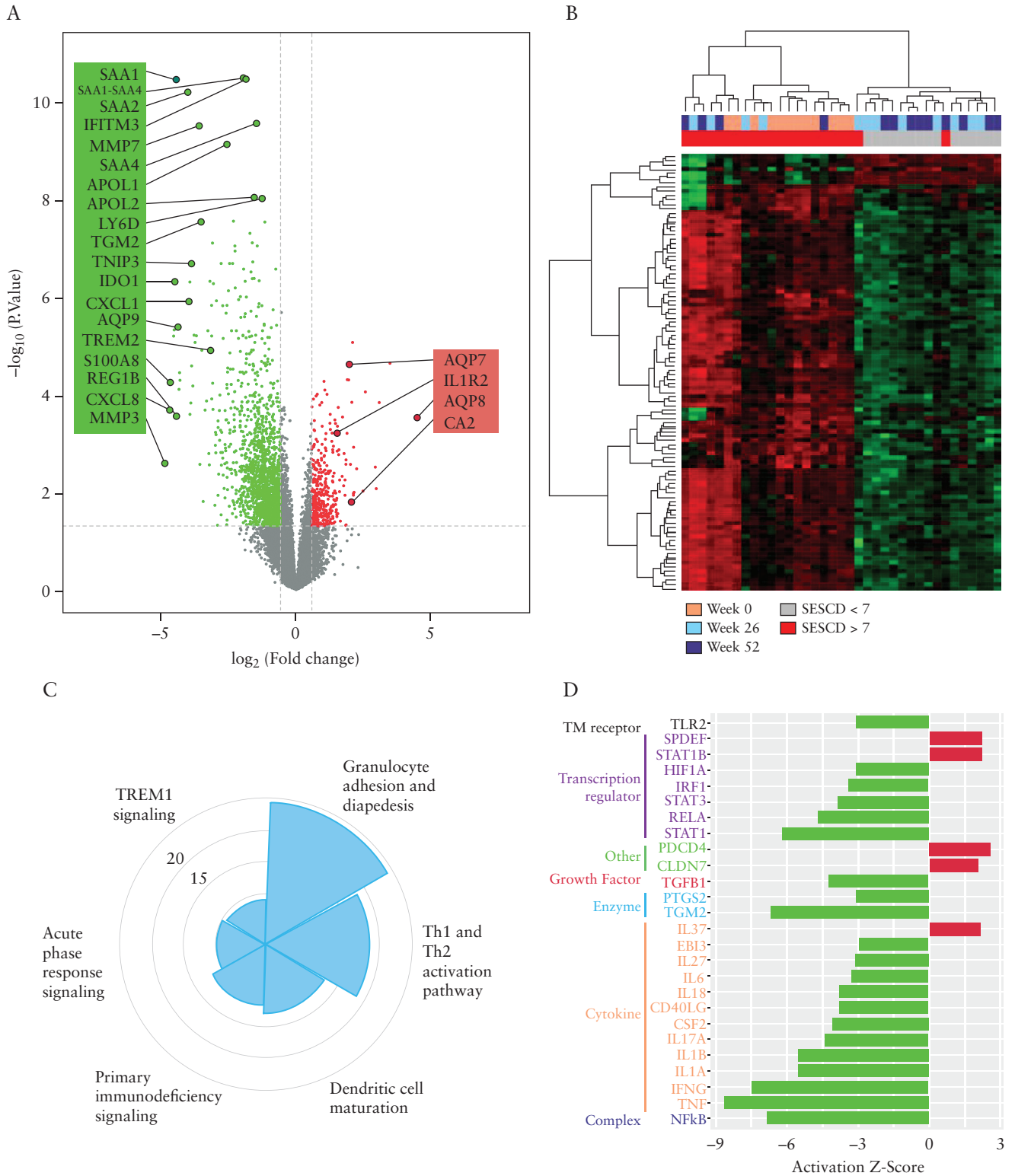
To better characterize the changes in the T-cell subsets following HSCT or anti-TNF $\alpha$ , we compared the expression of a selection of T-cell-related [Figure 8A–C] and myeloid-related [Figure 8D] transcripts as measured by RNAseq in both cohorts of patients. While expression of the neutrophil or 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<sub>RM</sub>] marker *ITGA1* were significantly regulated only in the HSCT-responding cohort. Two other T<sub>RM</sub> 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-TNF $\alpha$ - 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.

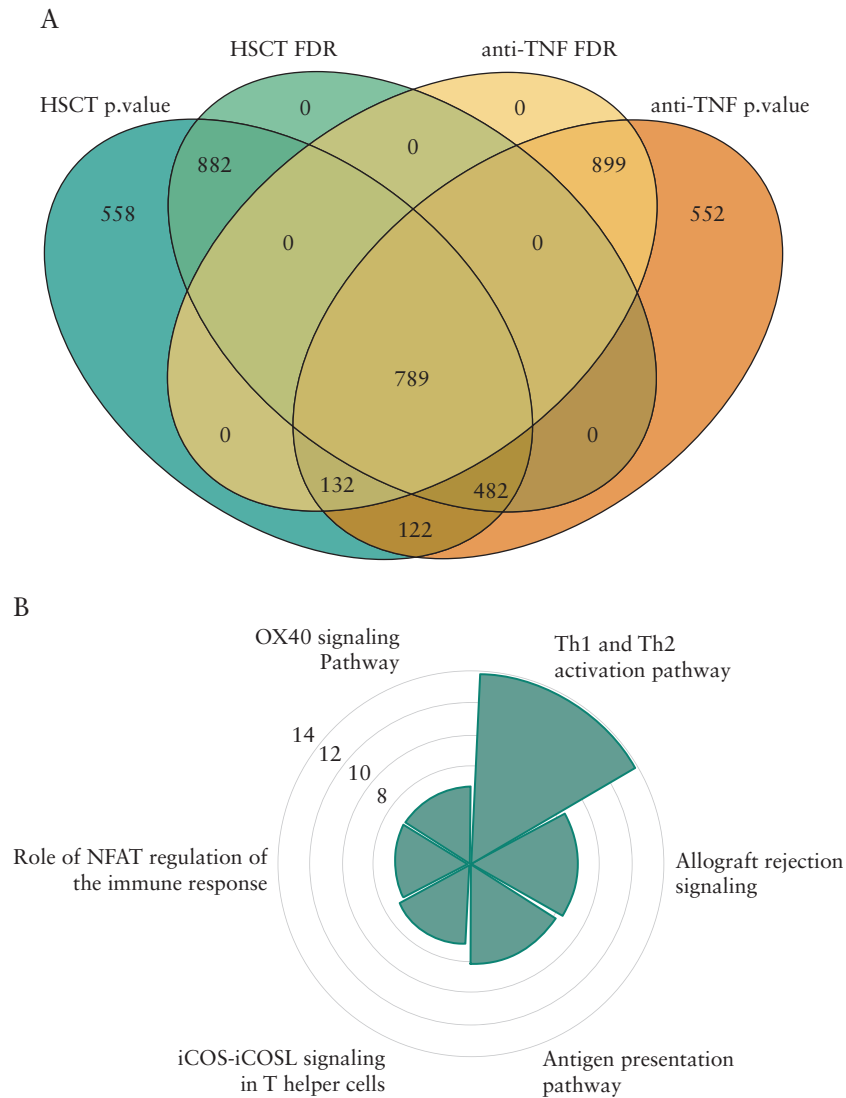
#### 4. Discussion

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.<sup>16,17</sup> Indeed, autologous HSCT has been shown to remodel the peripheral immune system, as evidenced by the regeneration of naïve B cells,<sup>11,18</sup> thymic reactivation,<sup>11,19–21</sup> renewal of the T-cell receptor repertoire<sup>11,19,21</sup> and the regeneration of regulatory T cells [Tregs]<sup>22,23</sup> 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  $\log_2(\text{fold change})$  is shown along the x-axis and the  $-\log_{10}(\text{corrected } p\text{-value})$  along the y-axis. Differentially expressed genes that reach significance [considered as corrected  $p < 0.05$  and  $|\text{FC}| > 1.5$ ] 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.

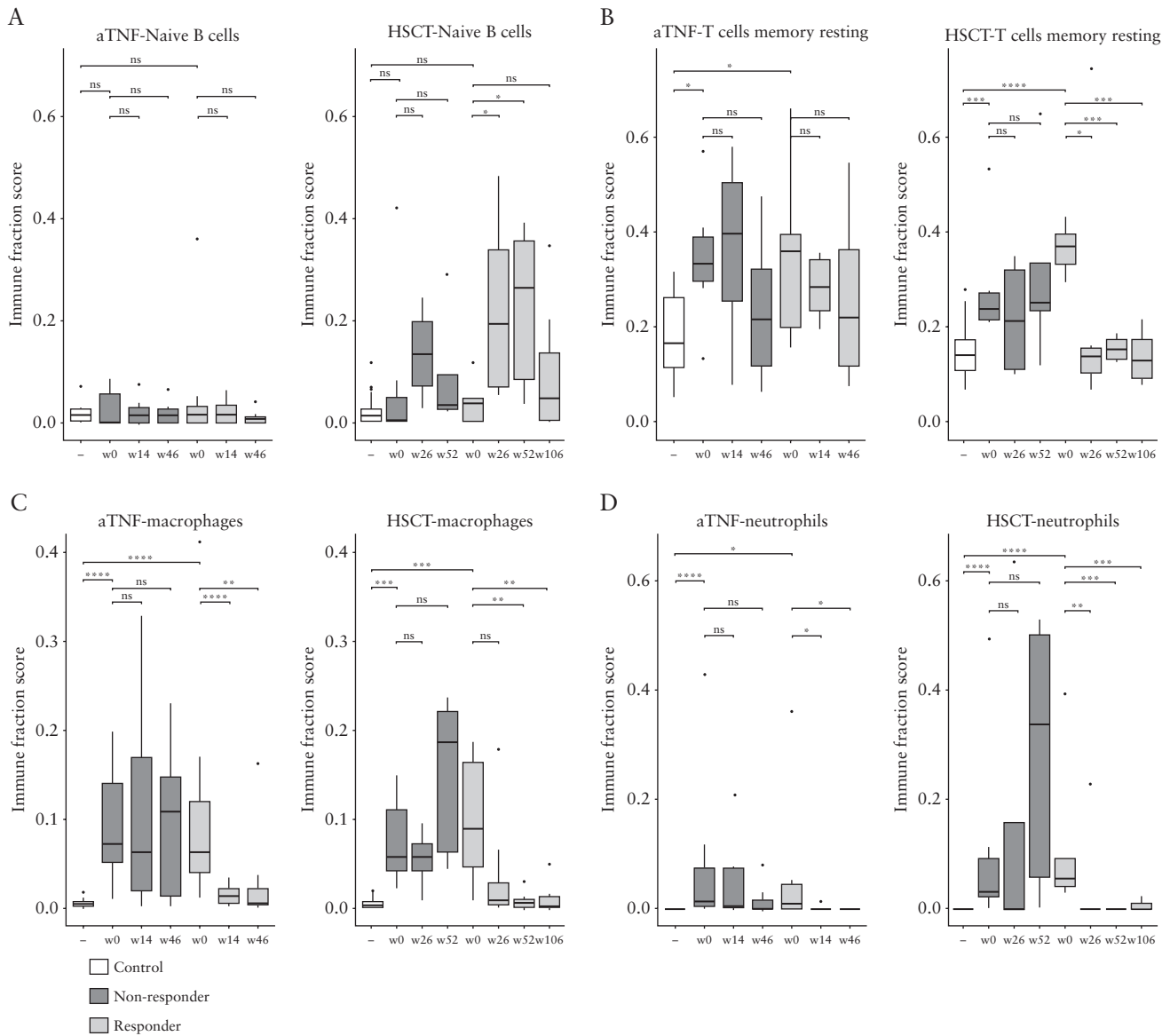


**Figure 6.** HSCT exclusive remission-induced gene signature is related to down-regulation of T-cell canonical pathways. [A] Venn diagram showing the number of genes that are differentially expressed in CD remitting colonic mucosa at week 52 after HSCT or week 46 after anti-TNF treatment compared to 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-TNF $\alpha$ . [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 analyze peripheral immune changes in a cohort of seven CD patients undergoing autologous HSCT.<sup>24</sup> In particular, the authors reported variations in the percentage of peripheral blood monocytes that produced TNF $\alpha$  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<sup>+</sup> T-cell compartment

remains compromised, while the CD8<sup>+</sup> T- and the B-cell subsets have completely recovered. Moreover, and in agreement with the existing literature,<sup>25</sup> naïve CD4<sup>+</sup> 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<sup>+</sup> memory compartment showed a delayed recovery, in agreement with the existing literature.<sup>26</sup> On the other hand, CD8<sup>+</sup> 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.<sup>26,27</sup> 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-TNF $\alpha$ . [A] Cell deconvolution analysis [CIBERSORT] of biopsy samples from healthy non-IBD controls [ $n = 19$ ] and CD patients before and after treatment with anti-TNF $\alpha$  [ $n = 22$ , 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.

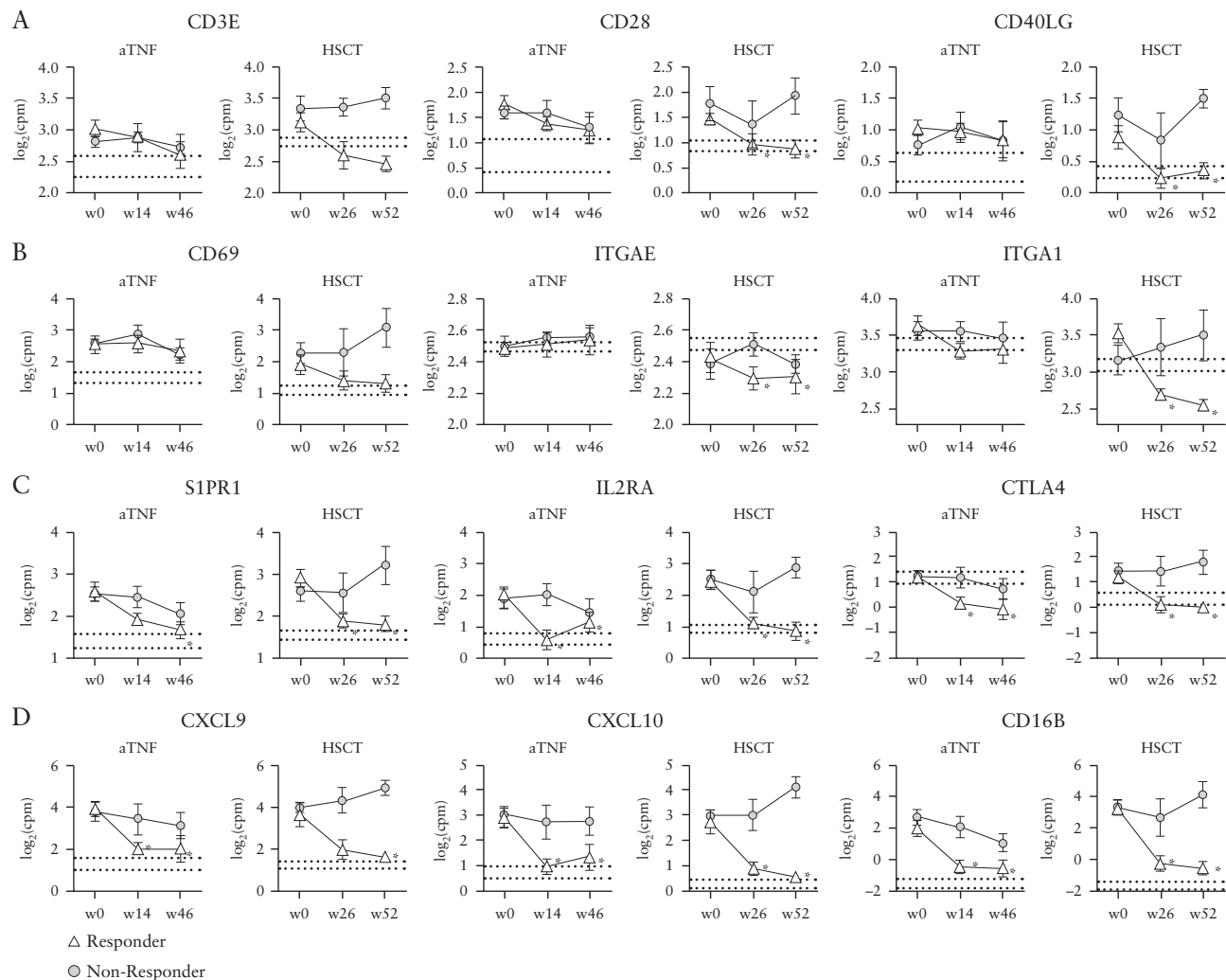
replenishment of the CD4 and CD8 compartments after HSCT has also been observed in MS patients.<sup>28</sup>

The rapid recovery and later expansion of the B-cell compartment we observed after HSCT was also in agreement with previous findings<sup>11</sup>; 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<sup>+</sup> reconstitution,<sup>29</sup> 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.<sup>11</sup> 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,<sup>29</sup> 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-TNF $\alpha$  and HSCT. Gene expression was determined in intestinal biopsies by RNAseq in CD patients treated with anti-TNF $\alpha$  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* [D]. 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 remodeling and regenerating pathways that have been extensively described to be modulated in response to conventional treatment.<sup>30,31</sup> 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-TNF $\alpha$  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-TNF $\alpha$  therapy was associated with changes in the neutrophil and M1 macrophage proportions in biopsies, changes in other immune cellular subsets, such as naive B cells or certain populations of T cells, were only observed after transplant.

The unique expansion of naive 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-TNF $\alpha$  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<sup>+</sup> memory compartment of patients in remission after HSCT. This population did not change to the same extent from anti-TNF $\alpha$  treatment, revealing a mechanism unique to the transplantation protocol. Anti-TNF $\alpha$  has been shown to partially modulate the mucosal T-cell repertoire, acting primarily on highly expanded clones.<sup>32</sup> 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-TNF $\alpha$  treatment.<sup>33,34</sup> While anti-TNF $\alpha$  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.<sup>32</sup>

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-TNF $\alpha$ , can act on the tissue-resident T-cell compartment [T<sub>RM</sub>], which constitutes a predominant T-cell subset in healthy mucosal tissues.<sup>35</sup> 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  $\alpha E$  [*CD103*] and  $\alpha 1$  [*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-TNF $\alpha$  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-TNF $\alpha$  cohort data. NP collected and analysed anti-TNF $\alpha$  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 are available at *ECCO-JCC* online.

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