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Short Report

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Restored Macrophage Function Ameliorates Disease Pathophysiology in a Mouse Model for IL10 Receptor-deficient Very Early Onset Inflammatory Bowel Disease

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Abstract

Background and Aims: Mutations in IL10 or the IL10 receptor lead to very early onset [VEO] inflammatory bowel disease [IBD], a life-threatening disease which is often unresponsive to conventional medication. Recent studies have demonstrated that defective IL-10 receptor signalling in innate immune cells is a key driver of severe intestinal inflammation in VEO-IBD. Specifically, IL10 unresponsiveness of macrophages, which govern the tight balance between pro- and anti-inflammatory responses in the intestinal system, plays a central role in the events leading to excessive inflammatory responses and the development of IBD.
Methods and Results: We here evaluated haematopoietic stem cell gene therapy in a VEO-IBD mouse model and demonstrated that the therapeutic response closely correlates with gene correction of the IL10 signalling pathway in intestinal macrophages. This finding prompted us to evaluate the therapeutic efficacy of macrophage transplantation in the *Il10rb*$^{-/-}$ VEO-IBD mouse model. A 6-week regimen employing a combination of depletion of endogenous hyperinflammatory macrophages followed by intraperitoneal administration of wild-type [WT] macrophages significantly reduced colitis symptoms.
Conclusions: In summary, we show that the correction of the IL10 receptor defect in macrophages, either by genetic therapy or transfer of WT macrophages to the peritoneum, can ameliorate disease-related symptoms and potentially represent novel treatment approaches for VEO-IBD patients.

Key Words: Cell and gene therapy; IL10 receptor; macrophages

1. Introduction

Macrophages have recently been highlighted as key players in tissue homeostasis in various organs.[1,2] In the intestinal system, macrophages cross-talk with T cells and other immune cells and govern the tight balance between pro- and anti-inflammatory responses.[3,4] Disturbances in this tightly balanced network can lead to the development of inflammatory bowel disease [IBD]. Previous studies have established an important role of IL10 signalling as a major regulator of immune tolerance in the intestinal mucosa. Mutations in IL10 or its receptor [IL10R] lead to IBD-like disease in mice and very early onset [VEO] IBD in humans, a severe disease affecting infants under the age of 1 year.[4–7] Infants with VEO-IBD are often unresponsive to conventional treatments and currently the only available curative treatment is allogeneic haematopoietic stem cell [HSC] transplantation, which is however associated with serious risks.[5,8]

Recent studies have demonstrated that defective IL10R signalling of innate immune cells is one key driver of severe intestinal inflammation in VEO-IBD. IL10R-deficient macrophages in the lamina propria [LP] show increased secretion of inflammatory cytokines coincident with decreased IL10 production, which ultimately impairs appropriate T cell responses and adversely affects the finely balanced immune homeostasis.[9,10] These data suggest that correction of the macrophage defect alone may have therapeutic implications.[11,12] In an effort to develop a novel treatment option for this patient group, we evaluated the therapeutic efficacy of HSC gene therapy for IL10R deficiency. Our findings support a central role of lamina propria macrophages in controlling inflammatory responses in this disease. Following on this observation, we analysed the therapeutic efficacy of adoptive macrophage transfer on VEO-IBD associated symptoms and pathology in an *Il10rb*$^{-/-}$ mouse model. The results demonstrate that restoring IL10 signalling using gene therapy or adoptive transfer of wild-type [WT] macrophages can mitigate the disease phenotype in this murine model of VEO-IBD. Our data indicate that targeting the malfunctional macrophage population alone can represent a novel therapeutic strategy in VEO-IBD.

2. Results

To evaluate the potential of HSC gene therapy for VEO-IBD due to mutations in the IL10 receptor, we used an established mouse model [*Il10rb*$^{-/-}$ 129SvEv IBD mice] carrying a deletion in *Il10rb* gene. This strain develops spontaneous and severe colitis at between 2 and 4 months of age.[6,7] To restore defective IL10 signalling, we employed a third- generation self-inactivating [SIN] lentiviral vector encoding the codon optimised murine *Il10rb* cDNA [PGK-*Il10rb*, Figure 1A]. Gene transfer of the *Il10rb* cDNA containing vector but not a control vector, expressing a green fluorescent protein [GFP] alone into *Il10rb*$^{-/-}$ murine lineage-depleted [lin$^-$] bone marrow cells, led to restoration of IL10-induced STAT3 phosphorylation upon IL10 stimulation in progenitor-derived macrophages [Figure 1B]. Transfer of gene-modified lin$^-$ cells into *Il10rb*$^{-/-}$ IBD mice was associated with a significant amelioration of weight loss comparable to animals receiving wild type [WT] HSCs, whereas animals that received control vector transduced HSCs uniformly showed a progressive weight loss over the observation period of 35 days following transplantation [Figure 1C]. Histological assessment of the colon revealed substantial variability in the histological scores between animals in the gene therapy group, and the trend towards improved histology scores was statistically non-significant [Figure 1D and E]. To better understand this heterogeneity in the treatment effect, we performed correlation analysis between gene marking in several haematopoietic lineages isolated from the lamina propria [LP] versus the therapeutic efficacy. This analysis revealed that the frequency of genetically corrected macrophages in the LP showed the highest correlation with histological scores [R^2 = 0.92, p = 0.0098]; in contrast, lymphoid gene marking did not correlate with the therapeutic efficacy [R^2 = 0.0267, p = 0.79] [Figure 1F].

These data, together with published data emphasising the role of macrophages in *Il10rb*$^{-/-}$ VEO-IBD,[9,10] suggest the hypothesis that correcting the macrophage population in the lamina propria could have a therapeutic effect on the colitis in VEO-IBD due to IL10R deficiency. Thus, we next evaluated the potential of macrophage-based cell therapy in *Il10rb*$^{-/-}$ mice. Macrophages were generated from bone marrow of wild-type mice by *in vitro* differentiation following a previously established protocol.[13,14] The resulting bone marrow-derived macrophages [BMDMs] showed characteristic adherence to non-tissue culture treated plastic dishes, a classical morphology in brightfield microscopy and cytospin preparations, and represented a highly pure population of >99% CD45$^+$/CD11b$^+$/F4/80$^+$ cells. Markers for dendritic cells [CD103], T cells [TCR], or B cells [B220] were uniformly absent [Supplementary Figure 1A and B, available as Supplementary data at *ECCO-JCC* online]. Adoptively transferred macrophages will likely encounter a niche which is occupied by endogenous macrophages in a highly inflammatory environment. Although compensatory feedback loops may result in increased expression of the anti-inflammatory cytokine IL10 in the colon of *Il10rb*$^{-/-}$ mice,[9,10,15] triggering the incoming macrophages to adopt an anti-inflammatory phenotype, we surmised that the pro-inflammatory environment could also lead to an activation of incoming macrophages, potentially aggravating the disease. In order to deplete the endogenous macrophage pool and partially reset the niche, we pre-conditioned the animals with clodronate liposomes.[15,16] A single intraperitoneal [i.p.] administered dose led to a strong reduction of CD45$^+$/CD11b$^+$/F4/80$^+$ macrophage populations in spleen, liver, peritoneum, and lamina propria 3 and 7 days following transplantation [Supplementary Figure 1C].

To evaluate the therapeutic efficacy of macrophage transfer in IBD, four experimental groups were included: untreated IBD mice; and IBD mice treated with [i] WT bone marrow-derived macrophages [BMDMs], or [ii] clodronate-liposomes [Clodro], or [iii] a combination therapy with clodronate liposomes and WT BMDMs [Clodro/BMDMs]. We used animals aged 3–5 months already showing clear signs of colitis. The BMDMs in the group receiving the combined treatment were administered 3–5 days following clodronate application by i.p. injection. We chose this local injection because peritoneal cavity macrophages are known to comprise a long-lived population of CD11b^{high}/F4/80^{high} tissue-resident macrophages [TRM] and, moreover, a reservoir of mature macrophages able to invade visceral organs and adapt a reparative phenotype.[17,18] We infused cells every 2 weeks for two or three treatment cycles which encompassed 4–6 weeks of observation [Figure 2A]. Animals sacrificed after 4–6 weeks of treatment showed improved colon morphology only in the Clodro/BMDM group as assessed by histological analysis. In contrast, animals treated with clodronate liposomes alone did not show a significant improvement of colon histology [Figure 2B and C]. Mice that received only macrophages showed even higher infiltration of inflammatory haematopoietic cells, and thus demonstrated higher histological scores than untreated animals.

We also analysed the composition of haematopoietic cells in the LP, including the immunophenotype of LP macrophages, by flow cytometric analysis [gating strategy in Supplementary Figure 2A, available as Supplementary data at *ECCO-JCC* online]. First, we observed the previously reported[9] large increase in MHCII$^+$/Ly6C^{high} LP inflammatory macrophages in IBD animals compared with WT

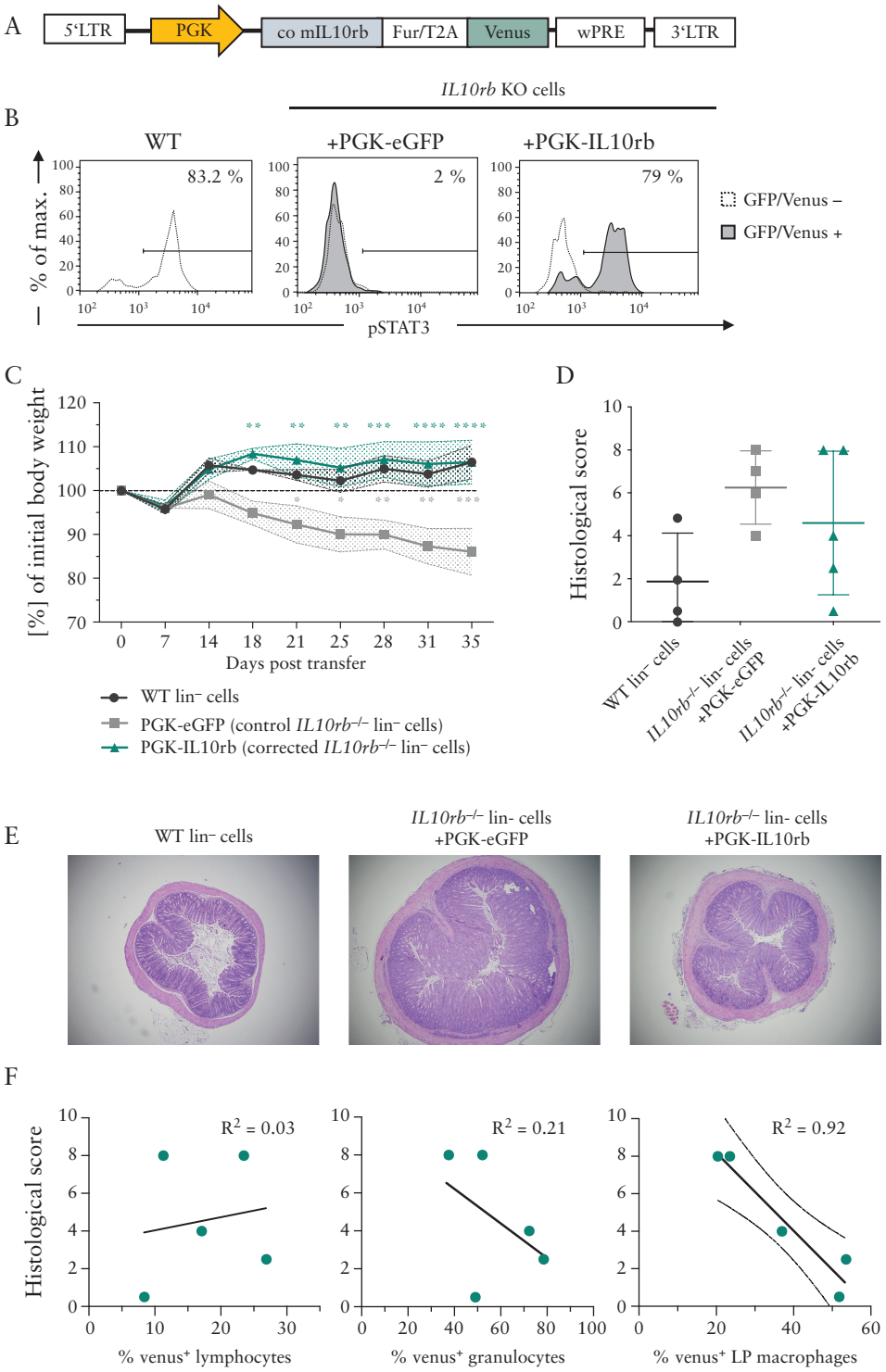

Figure 1. Therapeutic efficacy of haematopoietic stem cell [HSC] gene therapy in *Il10rb*$^{-/-}$ IBD mice correlates with gene marking in lamina propria macrophages. [A] A third-generation self-inactivating [SIN] lentiviral vector was used for gene transfer of a codon optimised [co] murine *Il10rb* cDNA coupled to the mVenus fluorescent reporter driven by the phosphoglycerate kinase [PGK] promoter. [B] Phosphorylation of STAT3 after stimulation with IL10 analysed by flow cytometry in macrophages derived from wild type [WT] or *Il10rb*$^{-/-}$ lin$^-$ bone marrow cells. *Il10rb*$^{-/-}$ lin$^-$ cells were transduced with a PGK-eGFP [control] or PGK-IL10rb therapeutic vector. [C] Control transduced *Il10rb*$^{-/-}$ lin$^-$ bone marrow cells, corrected *Il10rb*$^{-/-}$ lin$^-$ or WT lin$^-$ cells were transplanted into irradiated *Il10rb*$^{-/-}$ IBD mice and body weights were analysed for 35 days. Values are given as % of initial body weight (n = 4–5 per group, mean ± range [range depicted as shaded area]). [D] Quantification after histological scoring [n = 4–5 per group, individual values with mean ± SD]. [E] Representative light microscopy of H/E stained colon paraffin sections. [F] Correlation of the frequency of gene-marked lymphocytes, granulocytes, and macrophages in the lamina propria with the histological score [mean ± 95% CI]. LTR, long terminal repeat; wPRE, woodchuck hepatitis virus post-transcriptional regulatory element, significances were calculated by two-way ANOVA with Bonferroni's multiple comparison test, *p <0.05, **p <0.01, ***p <0.001, ****<0.0001, ns not significant. IBD, inflammatory bowel disease; SD, standard deviation; H/E, haematoxylin and eosin; CI, confidence interval; ANOVA, analysis of variance.

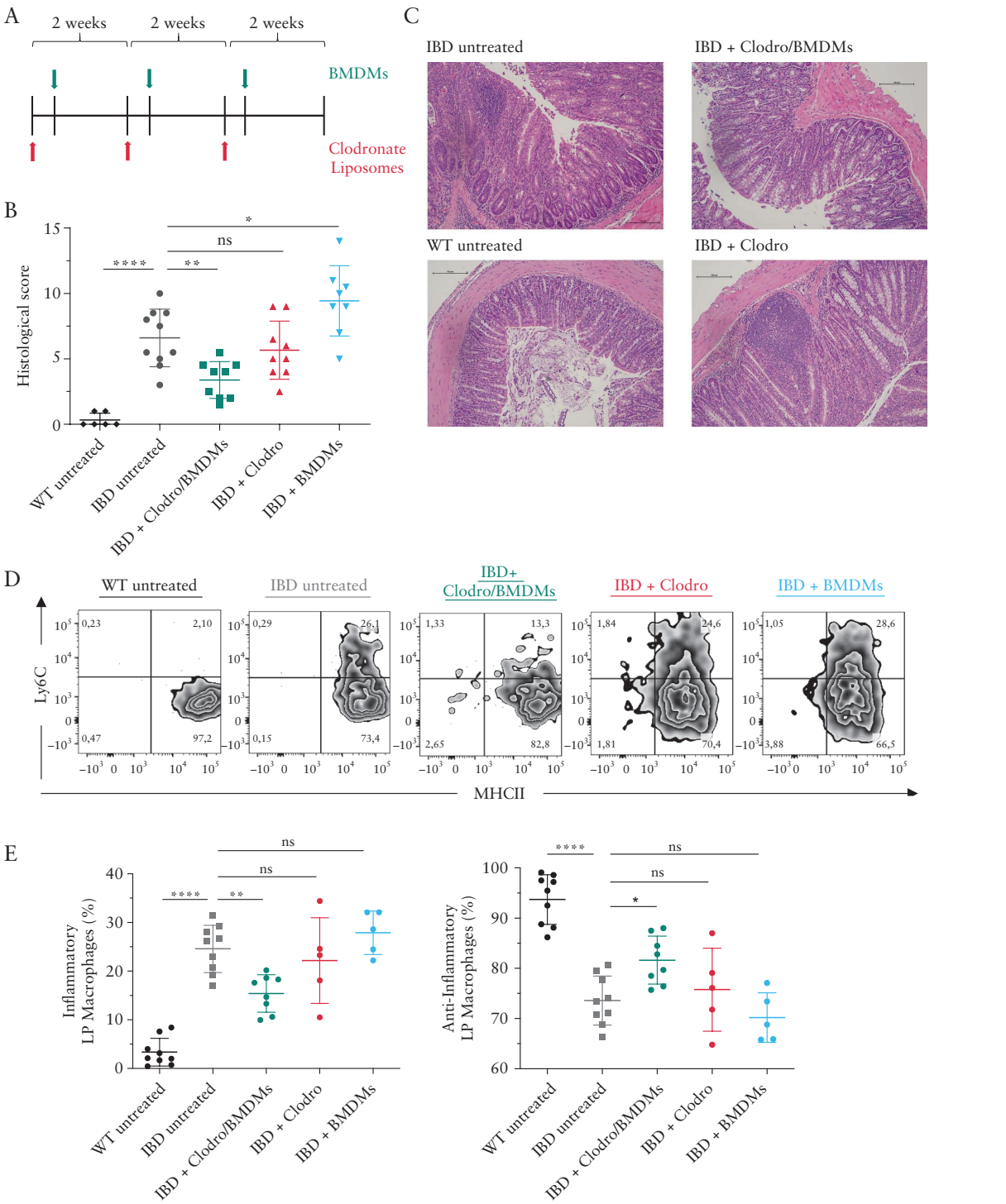

Figure 2. Macrophage transplantation reduces colitis in *Il10rb*$^{-/-}$ IBD mice. [A] Schematic representation of the experimental set-up. Macrophages were generated from WT BM cells and 5-10 x 10^6 cells were transplanted into IBD animals by intraperitoneal [i.p.] injection every 2 weeks [green arrows]. In some experimental groups, clodronate liposomes were administered i.p. [red arrows] to deplete endogenous macrophages before macrophage transplantation. The experiment comprised two to three treatment cycles. [B] Colon histology scoring was performed for untreated WT mice, untreated IBD animals or IBD animals treated two to three treatment cycles with clodronate liposomes [Clodro], bone marrow-derived macrophages [BMDMs], or a combination therapy of clodronate liposomes followed by BMDMs [Clodro/BMDMs]. Data represent five independent experiments, individual values with mean ± SD, $n = 8–10$ per group. [C] Representative light microscopy of H/E-stained colon paraffin sections. [D and E] Immuno-phenotyping of lamina propria [LP] macrophage populations [pre-gated on: CD45$^+$/CD11b$^+$/CD103$^-$/ Ly6G$^-$/CD64$^+$ monocyte/macrophages, see Supplementary Figure 2 for gating strategy, available as Supplementary data at *ECCO-JCC* online]. [D] Representative flow cytometry plots showing the frequency of Ly6C$^+$/MHCII$^+$ inflammatory and Ly6C$^-$/MHCII$^+$ anti-inflammatory LP monocyte/macrophages in different treatment groups; and [E] quantification of four independent experiments [individual values with mean ± SD, $n = 5–10$ per group]. Significances calculated by one-way ANOVA with Dunnett's multiple comparison test, *p <0.05, **p <0.01, ***p <0.001, ****<0.0001, ns not significant. IBD, inflammatory bowel disease; BM, bone marrow; SD, standard deviation; H/E, haematoxylin and eosin; WT, wild type; ANOVA, analysis of variance.

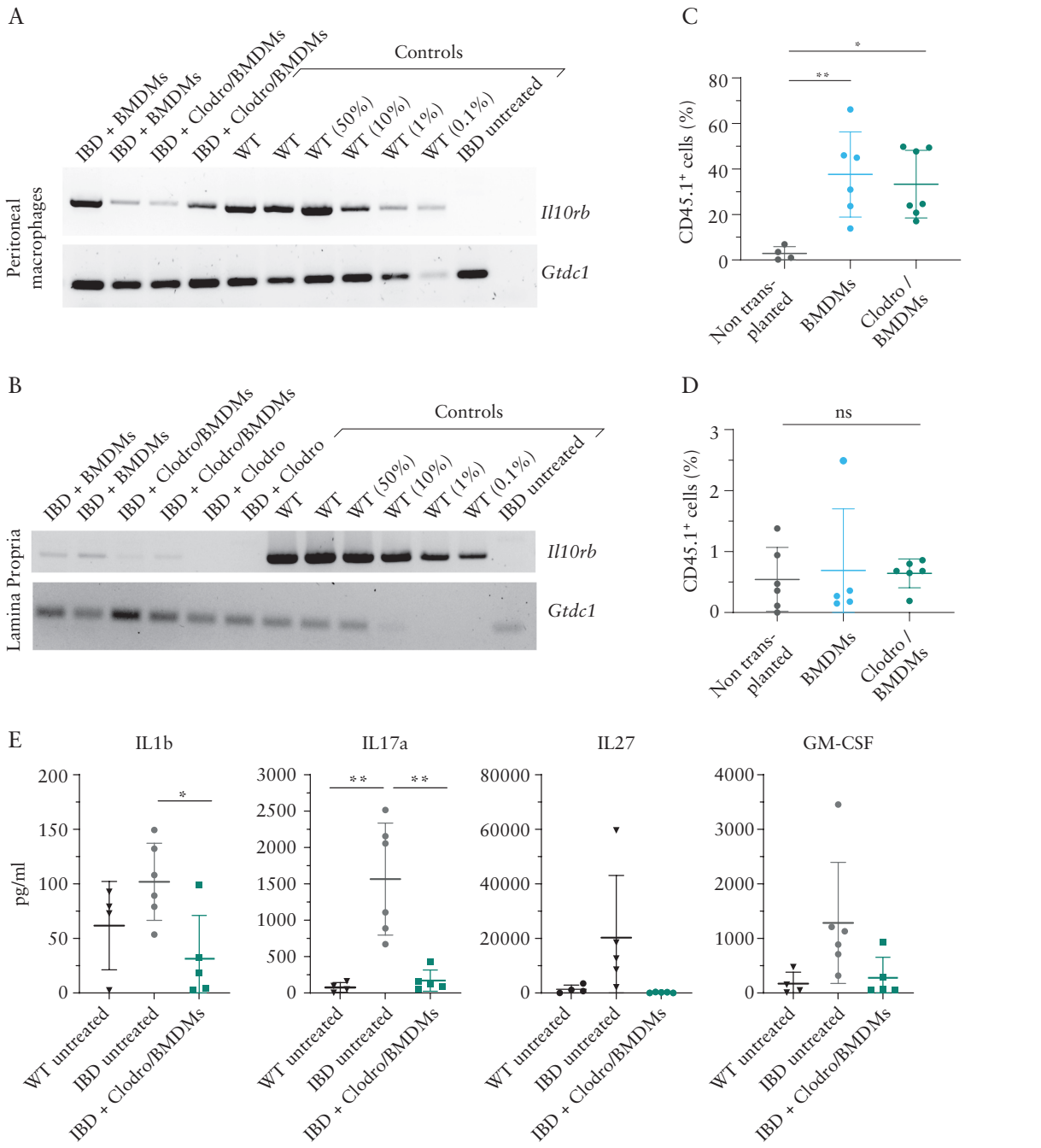

Figure 3. Detection of transplanted WT macrophages *in vivo* and cytokine profiling. [A and B] Polymerase chain reaction [PCR] was used to detect wild-type macrophages harbouring the *Il10rb* gene; glycosyltransferase-like domain containing 1 [*Gtdc1*] was used as a control in [A] peritoneal cavity macrophages and [B] cells isolated from the lamina propria of animals treated with: SV129 IL10rb$^{-/-}$ IBD animals treated with BMDMs, or clodronate liposomes, or a combination of clodronate liposomes and BMDMs, or clodronate liposomes only. Untreated IBD animals, dilutions of WT gDNA and H_20 are shown as controls [two individual animals/group are shown]. [C and D] Flow cytometry analysis of CD45.1$^+$ expression on donor-derived macrophages 6 days after transplantation into Bl6 Il10rb$^{-/-}$ IBD animals. Animals were either transplanted with BMDMs only or with a combination of clodronate liposomes and BMDMs [Clodro/BMDMs]. [C] Frequency of CD45.1$^+$ donor-derived cells among F480^{high}/CD11b^{high} peritoneal cavity macrophages; and [D] Frequency of CD45.1+ donor-derived cells among LP macrophages [mean ± SD]. [E] Secretion of IL1b, IL17a, IL27, and GM-CSF by macrophages derived from the peritoneal lavage fluid of different experimental groups [WT untreated, IBD untreated, and IBD+clodro/BMDMs] after LPS stimulation [data represent two independent experiments, individual values with mean ± SD, n = 4–6 per group, cells isolated after 4–6 weeks of treatment]. Significances are calculated by one-way ANOVA with Dunnett's multiple comparison test, *p <0.05, **p <0.01, ***p <0.001, ****<0.0001, ns not significant. IBD, inflammatory bowel disease; SD, standard deviation; WT, wild-type; LP, laminapropria; LPS, lipopolysaccharide; BBMDM, bone-marrow derived macrophages; ANOVA, analysis of variance.

animals [Figure 2D]. IBD animals treated with Clodro/BMDM demonstrated significantly reduced frequency of pro-inflammatory macrophages in the LP, an effect not observed in animals receiving a treatment with clodronate liposomes or BMDMs alone. Conversely, the frequency of anti-inflammatory LP macrophages was increased only in Clodro/BMDM-treated animals [Figure 2E]. Additionally, we found a trend towards lower expression levels of the pro-inflammatory cytokine IL1β in LP extracts only in Clodro/

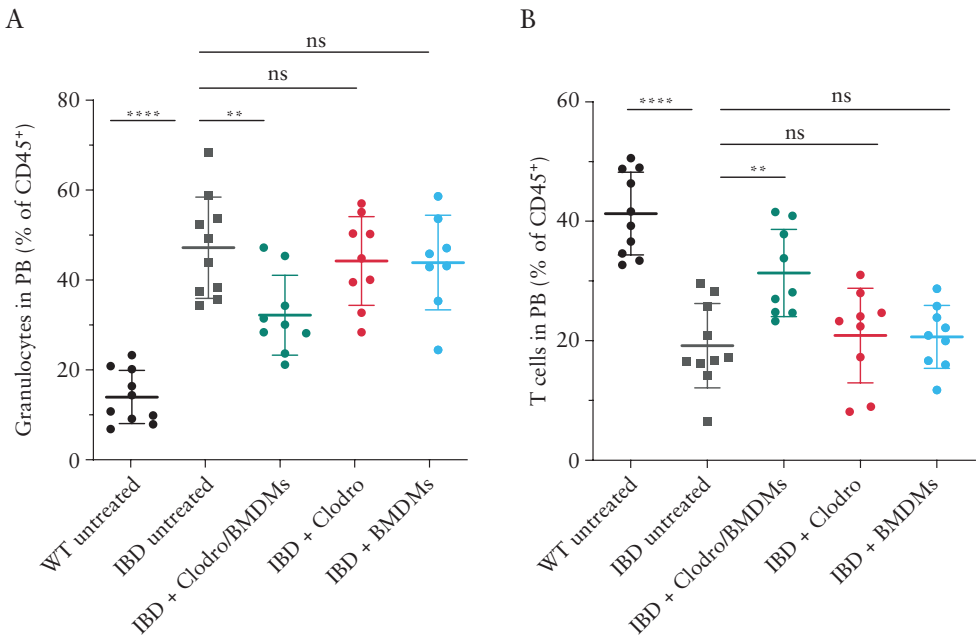

Figure 4. Macrophage-based therapy normalises peripheral blood cell composition in *Il10rb*$^{-/-}$ mice. Flow cytometric analysis was used to calculate the frequency of [A] granulocytes [CD45^{+}/CD3^{-}/B220^{-}/CD11b^{+}/Gr1^{high}] and [B] T cells [CD45^{+}/ CD11b^{-}/Gr1^{-}/CD3^{+}/B220^{-}] in peripheral blood [data represent five independent experiments, individual values with mean ± SD, n = 8–10 per group]. Significances calculated by one-way ANOVA with Dunnett's multiple comparison test, *p <0.05, **p <0.01, ***p <0.001, ****<0.0001, ns not significant. SD, standard deviation; ANOVA, analysis of variance.

BMDM -treated animals. IL17α was slightly reduced in Clodro/BMDM -treated as well as in animals treated only with clodronate [Supplementary Figure 2B], suggesting a modest effect from eliminating inflammatory macrophages from the LP environment.

To rule out IL10 receptor-independent effects of the transplanted macrophages in the combined treatment approach, we investigated the combination therapy of clodronate-liposomes with *Il10rb*$^{-/-}$ BMDMs. However, in this treatment group two out of three mice had to be sacrificed 3–5 days following the first macrophage treatment, due to severe systemic inflammation. When analysing the animals, we found a drastic increase in pro-inflammatory macrophages and decreased anti-inflammatory macrophages in the LP [Supplementary Figure 2C and D], indicating that the therapeutic effect of the combination treatment requires functional IL10R signalling in macrophages.

To further investigate the mechanism of this cell-based therapy, we analysed engraftment of the cells after transplantation. Polymerase chain reaction [PCR] of the *Il10rb* gene demonstrates the presence of WT cells in the peritoneal cavity of treated *Il10rb*$^{-/-}$ IBD mice [Figure 3A]. In contrast, very low frequencies of WT cells were detected in in the LP of transplanted animals, with a tendency towards slightly higher engraftment levels of WT cells in BMDM-only treated animals [Figure 3B], possibly related to increased migratory and chemo-attractant stimuli associated with ongoing inflammation. These findings were confirmed in a congenic *IL10rb*$^{-/-}$ mouse model on the Bl6 background. In Bl6 *Il10rb*$^{-/-}$ mice, which only develop mild colitis symptoms,[19] we could find the majority of transplanted CD45.1^{+} BMDMs among the F4/80^{high}/CD11b^{high} peritoneal macrophage population, whereas donor-derived cells could not be detected in the LP [Figure 3C and D]. These results indicate that transplanted macrophages affect the local milieu in the peritoneum and reduce inflammation without entering the LP in large numbers.

As previously described for BMDMs derived from *Il10rb*$^{-/-}$ mice and human macrophages derived from peripheral blood of *IL10R*$^{-/-}$ patients,[5,10] peritoneal macrophages from *Il10rb*$^{-/-}$ animals did not respond to a combined stimulation with IL10 and lipopolysaccharide [LPS] with the typical morphological changes of WT cells and failed to downregulate the secretion of pro-inflammatory cytokines such as IFNγ, IL6, or IL17α [Supplementary Figure 3A and B, available as Supplementary data at *ECCO-JCC* online]. Importantly, when analysing peritoneal macrophages isolated from the animals that received combination treatment with clodronate liposomes and WT BMDMs, we observed a significant reduction in IL17α and IL1β secretion as well as a trend towards lower levels of IL27 and GM-CSF secretion after LPS stimulation, when compared with cells isolated from untreated IBD animals [Figure 3E]. Similar observations were made in the Bl6 *Il10rb*$^{-/-}$ mouse model. Interestingly, only macrophages isolated from the Clodro/BMDM groups showed a normal response to LPS treatment and low levels of IL17α, IL1β, IL27, or GM-CSF secretion, whereas macrophages from the BMDM-only group responded with a higher secretion of those cytokines [Supplementary Figure 4, available as Supplementary data at *ECCO-JCC* online]. Together, these data show that the transplanted WT macrophages persist in the peritoneal cavity, and that only after previous clodronate treatment do transplanted macrophages induce a normalised cytokine response to an inflammatory stimulus and thus ameliorate the chronic inflammation in IBD mice.

Interestingly we not only observed phenotypic and cellular changes in colon tissues of successfully treated mice, but also changes in the composition of the peripheral blood. *Il10rb*$^{-/-}$ IBD mice had increased granulocyte and reduced T cell frequencies in their peripheral blood, which is a hallmark of diverse chronic inflammatory conditions.[20,21] In IL10-signalling-deficient mouse strains, this metric can be used as a surrogate read-out for colitis onset. The myeloid lineage bias was significantly reduced only in animals treated with a combined therapy; it correlated well with the amelioration of colon inflammation, and no systemic effect on haematopoiesis was observed in other treatment groups [Figure 4A and B].

3. Discussion

We here demonstrate that restoring macrophage function either by HSC gene therapy or by local adoptive macrophage transfer of wild-type cells to the peritoneum combined with depletion of endogenous hyperinflammatory macrophages, can ameliorate colitis and systemic inflammation in an *Il10rb*$^{-/-}$ mouse model for VEO-IBD. HSC gene therapy could provide a permanent cure, but careful assessment of potential side effects related to recombinant *IL10rb* expression is needed. Ectopic or dysregulated expression of the transgene may induce aberrant signalling in cell types which are normally not responsive to IL10, with unknown physiological consequences.

Importantly, the genetic IBD model used in this study mimics the complex immune phenotype of VEO-IBD and more accurately reflects the human disease, as compared with chemically induced colitis models such as dextran sulphate sodium [DSS]-induced colitis. In contrast to DSS-induced models of colitis, the use of *Il10rb*$^{-/-}$ mice allowed us to investigate a therapeutic intervention in mice with a colitis established for several months and with a longer observation period than is possible in the chemically induced model. Our findings provide additional support for recent reports highlighting the importance of macrophages in intestinal homeostasis, and support a therapeutic concept in which the intestinal immune microenvironment may be re-educated via modulation of macrophages to combat inflammatory conditions.[11,22]

Interestingly, we did not observe significant therapeutic efficacy using a monotherapy with BMDMs or clodronate liposomes. BMDM transfer alone actually showed a trend towards a more severe phenotype, which is consistent with a model in which the inflammatory environment in *Il10rb*$^{-/-}$ IBD mice triggers the migration and repolarisation of the transplanted macrophages to a pro-inflammatory phenotype. This highlights the necessity to deplete the defective endogenous peritoneal and lamina propria macrophages before transplantation. However, it will be important to investigate in future studies whether other pre-conditioning regimens, such as cytokine or cell type-specific antibodies or anti-inflammatory drugs, can replace the clodronate regimen, and at which intervals the depletion of the endogenous macrophage pool in the peritoneal cavity and the lamina propria is necessary for therapeutic efficacy. The microbicidal activity of transplanted macrophages may also be a contributing factor to the phenotypic improvements in our mouse model, although likely a minor one, as suggested by the low cell frequency found in the lamina propria and the need for clodronate depletion before cell injection.[23] A biological approach which could obviate the need for conditioning and enhance efficacy is the transfer of defined, *in vitro* polarised anti-inflammatory macrophage subsets that retain their cell state even when exposed to a repolarising milieu *in vivo*. This strategy could also benefit patients with the frequently occurring adult-onset forms of IBD, potentially without the need for a repetitive therapy because the patient's Ly6C^{+} monocytes recruited to the LP express the IL10 receptor and are able to adopt an anti-inflammatory phenotype after the inflammation is resolved.

Prospectively, the adoptive transfer of anti-inflammatory macrophages in VEO-IBD might help to stabilise young patients who are unresponsive to conventional therapy, until a suitable bone marrow donor is available. In addition, autologous HSC gene therapy, which would eliminate the need for donor search, the risk of graft-versus-host-disease (GVHD), and graft rejection, could after further development generate a constant source of corrected macrophages *in vivo* and thus would represent a practical translational approach to this disease.

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Conflict of Interest

The authors have declared that no conflict of interest exists.

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Author Contributions

MA and CB designed the study, performed experiments, analysed data, wrote the manuscript, and obtained funding. A Mucci, A McCabe, SF, and KW performed experimental work and analysed data. SS, NL, and DAW provided funding, conceptual advice, discussed results, and critically revised the manuscript.

Data Availability

All data are incorporated into the article and its Supplementary material, available at *ECCO-JCC* online. Further primary data are available on reasonable request to the corresponding author.

Supplementary Data

Supplementary data and methods are available at *ECCO-JCC* online.

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