

Supplemental information

**Developmental role of macrophages
modeled in human pluripotent stem
cell-derived intestinal tissue**

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Supplemental Text and Figures

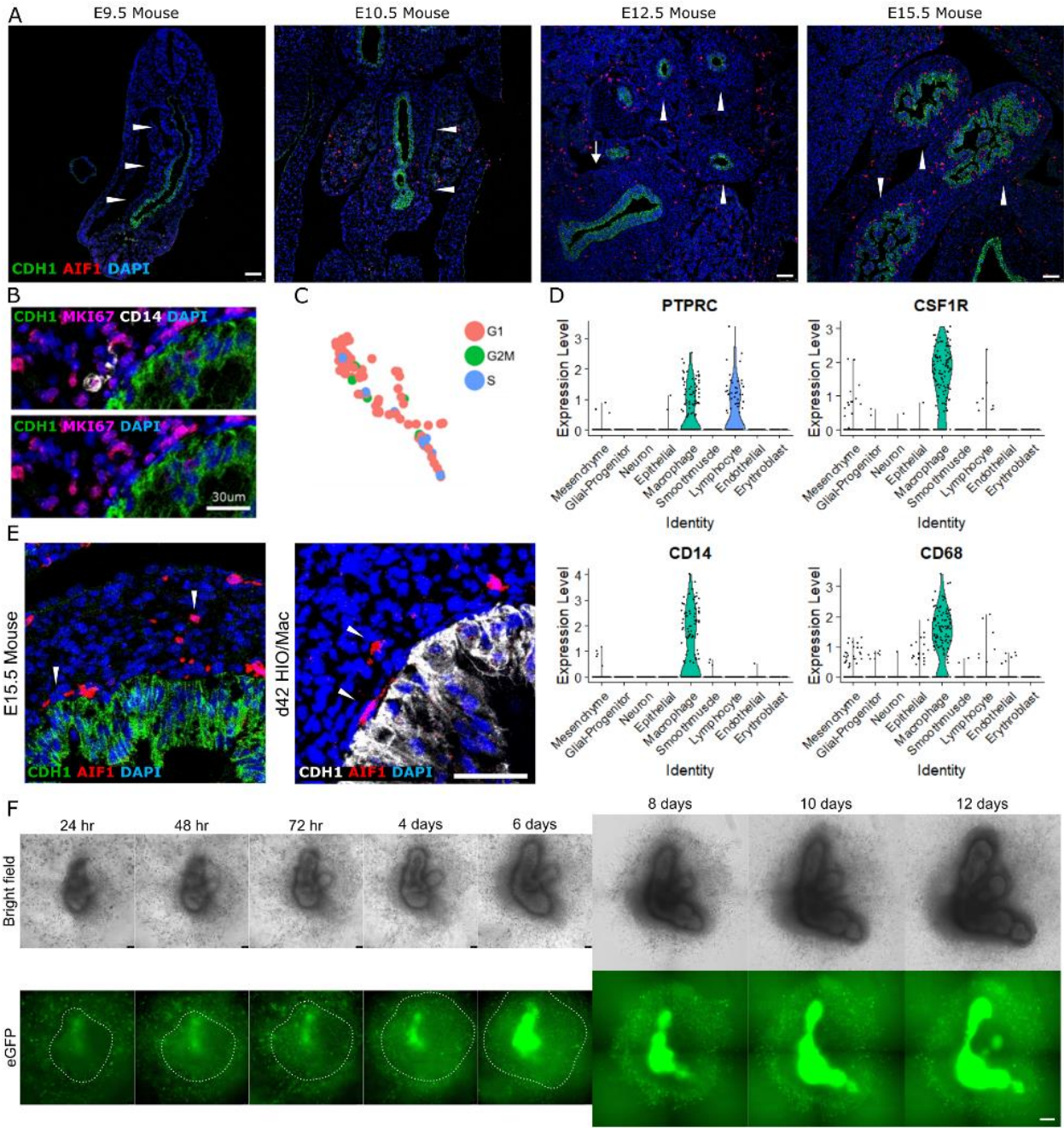


Figure S1. Macrophages in early embryonic mouse intestine, fetal intestine, and human intestinal organoid (HIO), related to Figure1 & 2 & 4.

- (A) Representative immunofluorescence images of mouse embryonic intestine for macrophages (AIF1), epithelium (CDH1), and nuclei (DAPI) at four time points of development. Arrowhead, mid-hindgut. Arrow, Foregut.
E9.5, n=4, E10.5, n=6, E12.5, n=6, E15.5, n=6. Scale bar = 75µm.
- (B) Representative immunofluorescence image of day 42 HIO/Mac for epithelium (CDH1), proliferation (MKI67), macrophages (CD14), and nuclei (DAPI). Scale bar = 30µm.
- (C) Cell cycle status of macrophages in day 37 HIO/ENS/Mac (C1/C2/C5) from scRNAseq.
- (D) Violin plot of gene markers used to identify the unsupervised cluster as macrophage in scRNAseq.
- (E) Representative immunofluorescence images for macrophages (AIF1), epithelium (CDH1), and nuclei (DAPI) in E15.5 mouse intestine and day 42 HIO/Mac. Arrowhead, macrophages localized at adjacent to epithelium and within mesenchyme. Scale bar = 50µm.
- (F) Bright field and GFP fluorescence images of human intestinal organoid (HIO) and macrophage co-culture over the course of 12 days. Macrophages were derived from hiPSC^{eGFP}. Scale bar = 150µm.

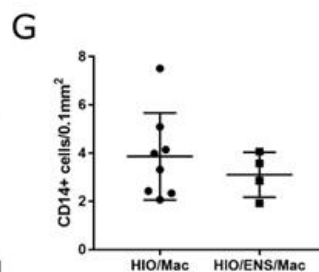
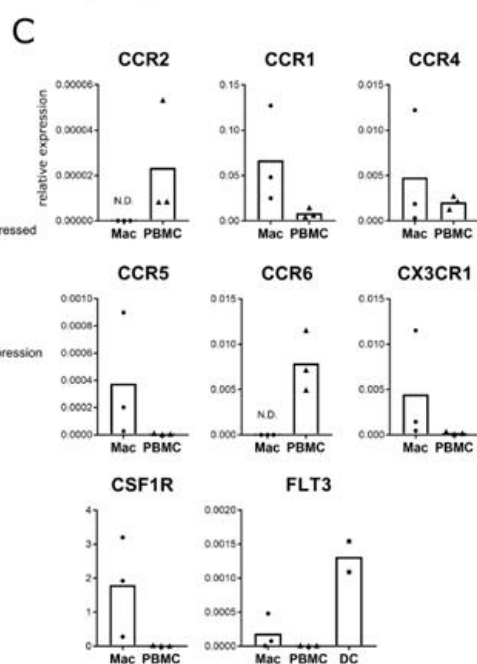
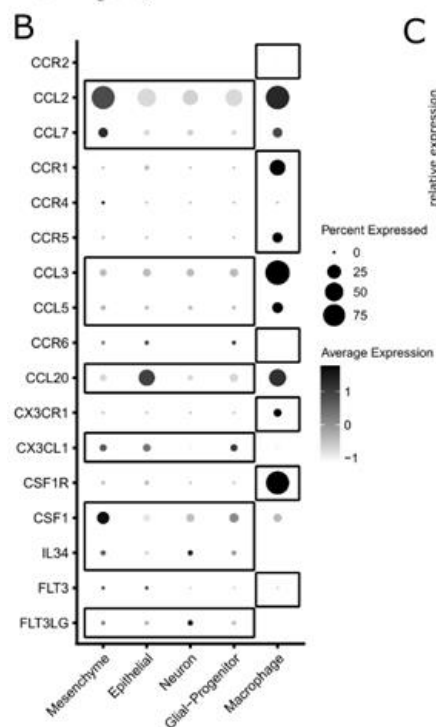
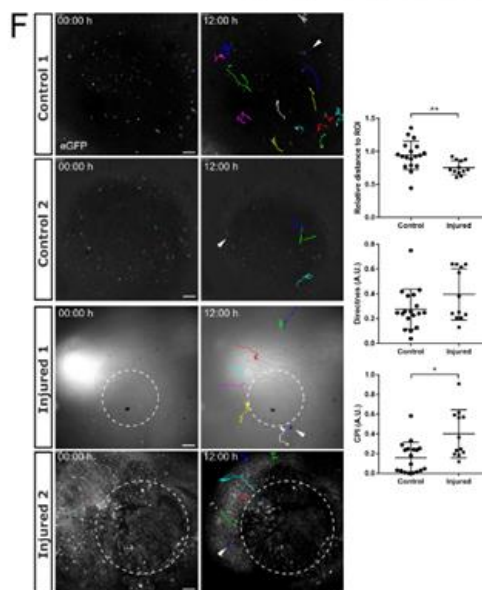
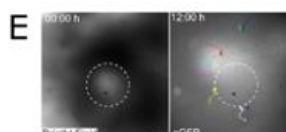
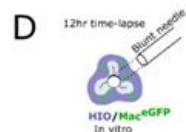
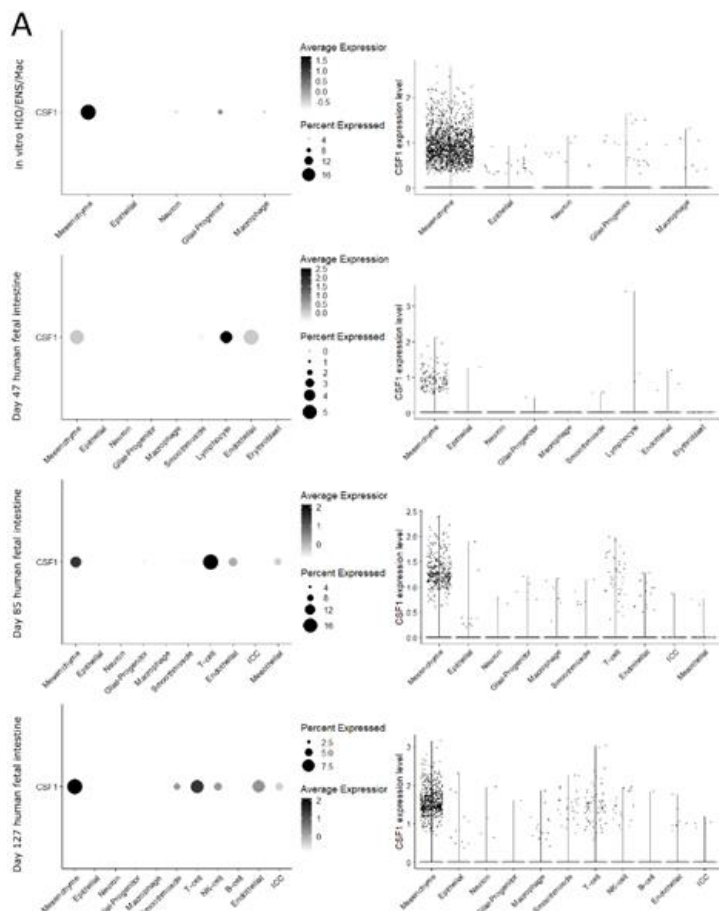


Figure S2. Migration and retention of macrophages in the organoid, related to Figure1 & 2.

- (A) Dot plots and violin plots of *CSF1* expression in presumptively annotated unsupervised clusters of the day 37 organoid, day 47, day 85, day 127 fetal intestine datasets. Dot plots: Percent Expressed, percentage of cells within the cluster expressing the gene. Violin plot: Each dot represents a cell.
- (B) Dot plot of ligand and receptor involved in macrophage recruitment in scRNAseq of HIO/ENS/Mac (C1/C2/C5).
- (C) qPCR of receptor genes for hiPSC-derived macrophages (Mac [C2, C3, C5]) prior to co-culture with HIO. Peripheral blood mononuclear cell (PBMC) and dendritic cell (DC) used as a reference previously shown to express the genes. Normalized to GAPDH expression. Mac, n = 3, PBMC, n = 3, DC, n = 2, biological replicates.
- (D) Schematic of the *in vitro* organoid puncture injury.
- (E) Demarcation of the injury as indicated by the bright field image and the corresponding hiPSC^{eGFP}-derived macrophages (Mac^{eGFP}) tracing.
- (F) Representative GFP fluorescence images from the 12-hour time lapses of control and injured *in vitro* HIO/Mac^{eGFP} (C1/C4). Colored lines and dots: tracing of the macrophage location over time. Dotted circle: region of the injury. Arrowhead: stationary points in the organoid to track the drifting of the entire sample. To the right: Quantification of relative distance of the macrophages, at 12 hour vs. 0 hour, from the center of the injury (Injured) or an arbitrary point within the image determined blinded (control). A measure of straightness of cell trajectories quantified as directness. Degree of directed migration towards a region of interest quantified as chemotactic precision index (CPI). See STAR Methods for calculation. Control, n = 2 organoids, n = 18 cells, Injury, n = 2 organoids, n = 12 cells, each data point represents a cell. Mean & s.d. (d-f). p = 0.0037, Welch's t-test (d). p = 0.2486 (e), p = 0.0116 (f), Wilcoxon rank sum test (Mann-Whitney). Scale bar=100µm.
- (G) Quantification of macrophage numbers within day 42 HIO co-cultured only with macrophage or with macrophage and vagal neural crest cells (ENS precursor). CD14-positive macrophages counted in non-epithelial region of the organoid (CDH1-negative & DAPI-positive) cryosections. Each data point represents an organoid. Result of two experiments. iPSC lines: HIO=C1, ENS precursor=C1, macrophage=C2. Related to Figure 2F. HIO/Mac: n = 8. HIO/ENS/Mac: n = 4. Mean & s.d. P = 0.4577, student's t-test.

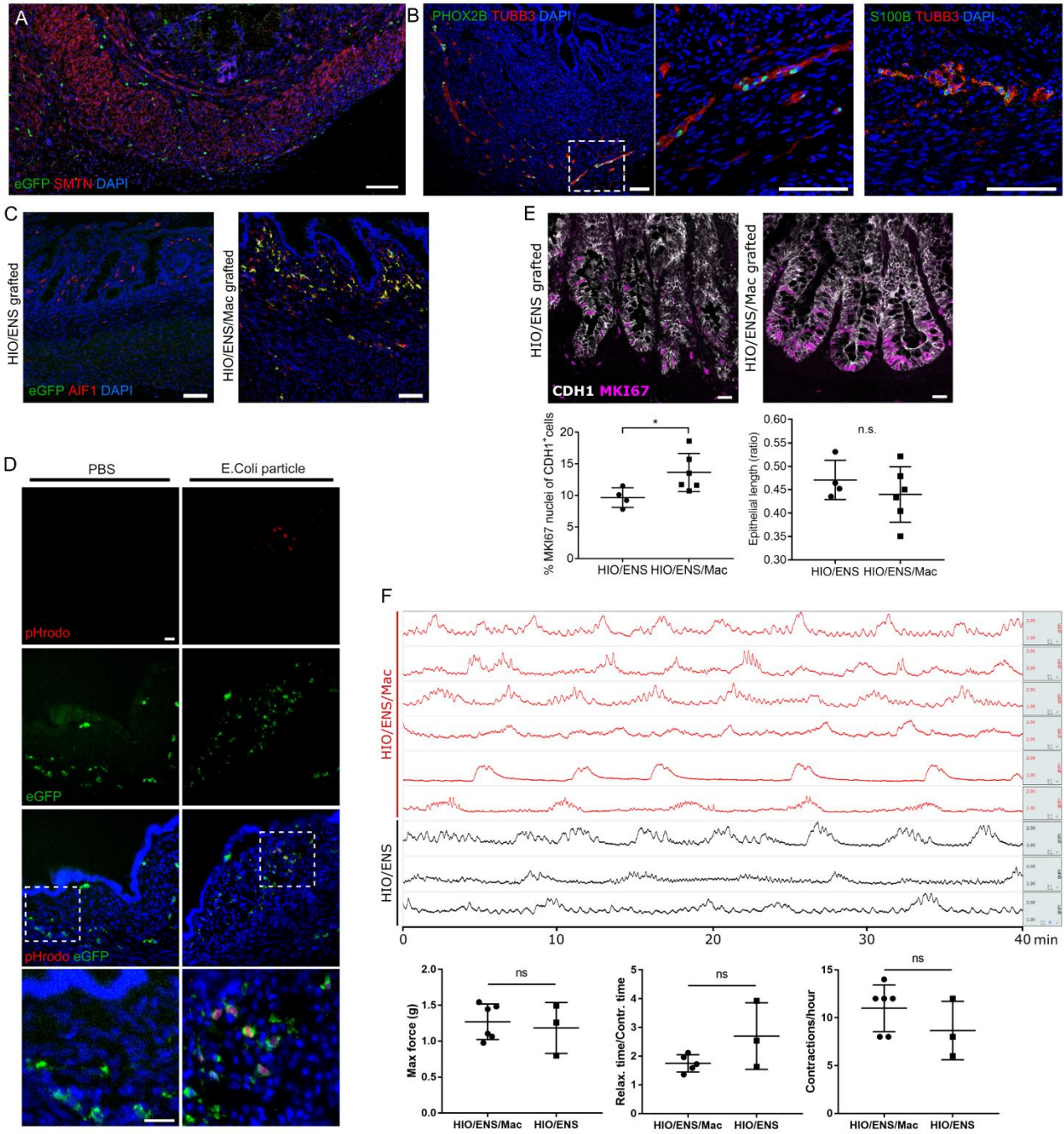


Figure S3. Characterization of grafted organoids, related to Figure 3.

- (A) Representative immunofluorescence image of 10 week grafted HIO/ENS/Mac^{eGFP} probed for macrophages (eGFP), smooth muscle (SMTN). Nuclei (DAPI). Scale bar=100μm.
- (B) Representative immunofluorescence image of enteric neurons (PHOX2B, TUBB3) and glial cells (S100B) in 10 week grafted HIO/ENS/Mac. Nuclei (DAPI). Scale bar=100μm.
- (C) Representative immunofluorescence images of grafted organoids combined without (HIO/ENS) or with hiPSC^{eGFP} derived macrophages (HIO/ENS/Mac^{eGFP}). Probed for iPSC-derived macrophages (eGFP) and macrophages (AIF1). Scale bar=50μm.
- (D) Tissue sections of grafted HIO/Mac^{eGFP} (C1/C4) injected with pHrodo-E.Coli particle conjugates or PBS (vehicle) into the lumen. Tissue sections counterstained with DAPI. Internalized E.Coli signals were only found in flat epithelial areas. PBS control: n=3. pHrodo-E.Coli particle: n=2. Scale bar=25μm.
- (E) Representative Immunofluorescence images of the crypt for proliferating (MKI67) epithelial cells (CDH1) and the quantification of MKI67-positive epithelial cells and the length of epithelium (epithelial length/total tissue thickness). Samples from two derivations/experiments. HIO/ENS (C1/C2): n = 4. HIO/ENS/Mac (C1/C2/C3 and C1/C2/C5): n = 6. p=0.0264. p=0.3961. Welch's t-test. Student's t-test. Mean & s.d. Scale bar=25μm.
- (F) Isometric force measurements of grafted organoids with or without combined macrophages. Each row represents an individual grafted organoid sample. Quantification of maximal force recorded, ratio of time required for relaxation to contraction, and frequency of contraction. See STAR Methods. HIO/ENS/Mac (C1/C2/C3), n = 6, HIO/ENS (C1/C2), n = 3. p = 0.7303, p = 3252, p = 0.2904, Welch's t-test. Mean & s.d.

Figure S4. scRNAseq analyses of fetal intestine and organoid macrophages, related to Figure 4.

- (A) Heatmap of all 77 upregulated genes in fetal intestinal macrophages between day 127 and day 80.
- (B) Gene ontology annotations of differentially expressed genes from for commonly upregulated genes between fetal intestinal and organoid macrophages and upregulated only in fetal intestinal macrophages.
- (C) Gene Set Enrichment Analysis (GSEA). Top 20 biological process (by NES) enriched in each fetal intestinal or organoid macrophages. FDR q-value cut off < 0.25 .
- (D) Volcano plots of differentially expressed genes in fetal intestinal macrophages between day 127 vs. day 47, day 101 vs. day 59, day 132 vs. day 85. Threshold of discovery (dotted line), \log_2 fold change > 1 , adjusted p-value < 0.001 , Wilcoxon rank sum test.

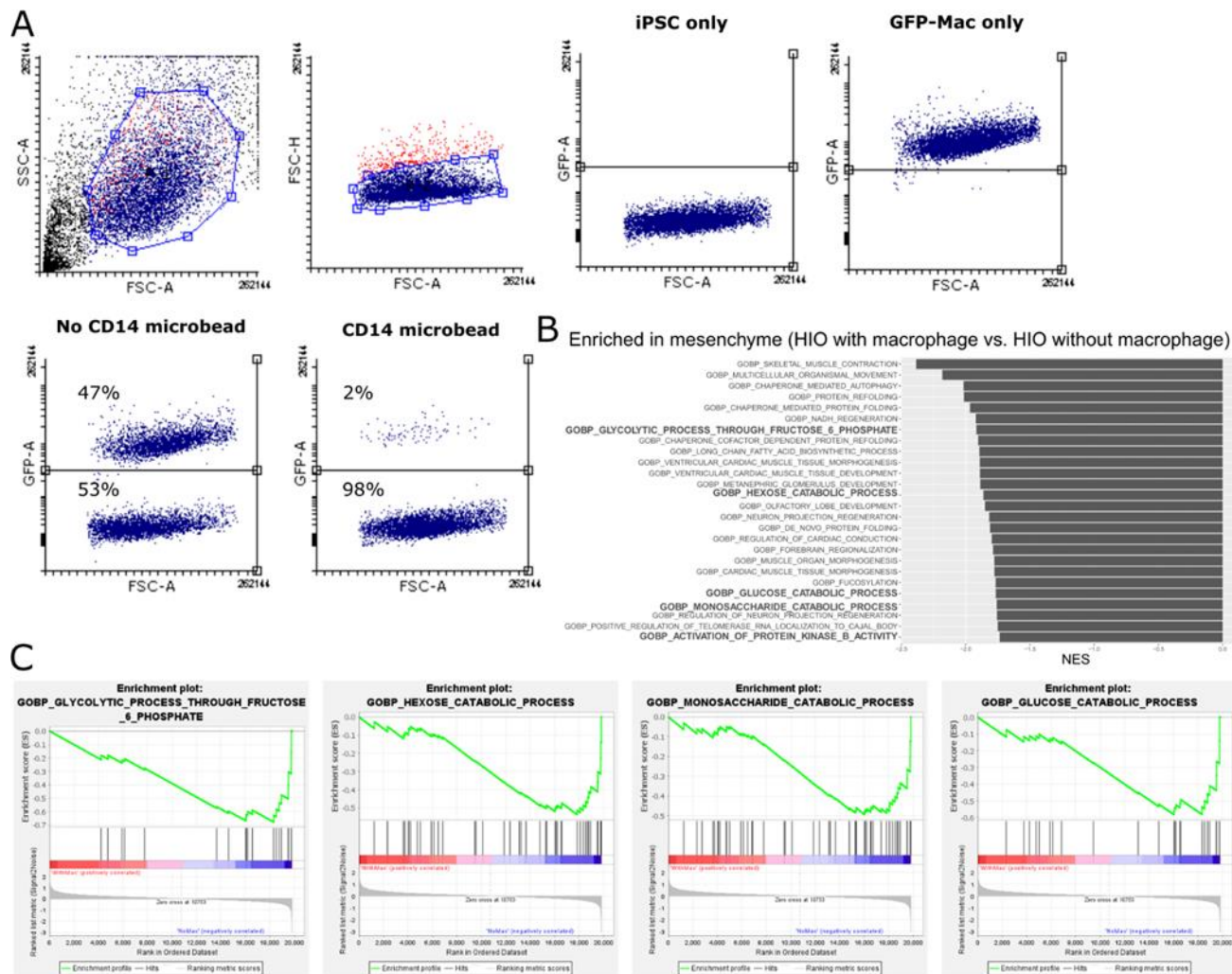


Figure S5. Efficiency of macrophage removal using magnetic microbead, related to Figure STAR Methods & Figure 6.

- (A) Efficiency of macrophage removal with microbead. Left, gating for cytometry of 1:1 cell mix of hiPSC^{WT} and hiPSC^{eGFP}-derived macrophage. Right, percent GFP-positive cells with only hiPSC^{WT} or only hiPSC^{eGFP}-derived macrophage. Bottom, percent GFP-positive macrophages of the cell mix either without or with anti-CD14 microbead mediated macrophage removal.
- (B) Gene Set Enrichment Analysis (GSEA). All 26 significant biological processes enriched from comparing mesenchymal cells from HIO or HIO/Mac. FDR q-value cut off < 0.25.
- (C) Enrichment plots of associated with glucose metabolism from (B).

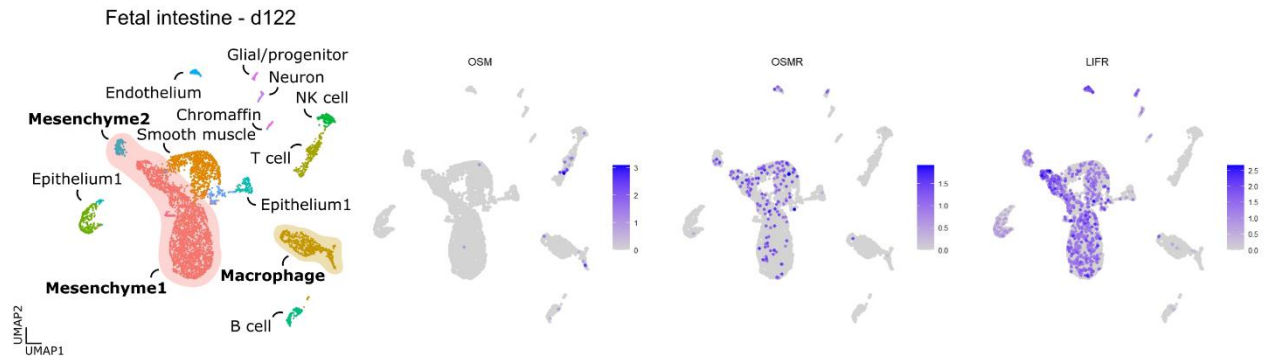
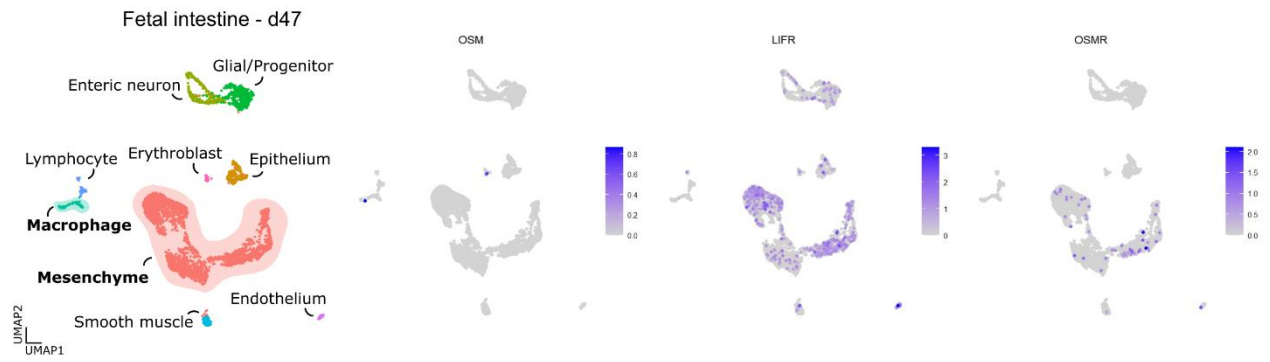


Figure S6. Expression of OSM, LIFR, and OSMR in the fetal intestines, related to Figure 7.

UMAP plot of day 47 and day 122 fetal intestine scRNAseq datasets and corresponding plots showing gene expression of OSM, LIFR, and OSMR.

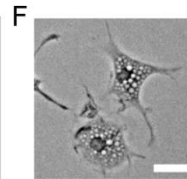
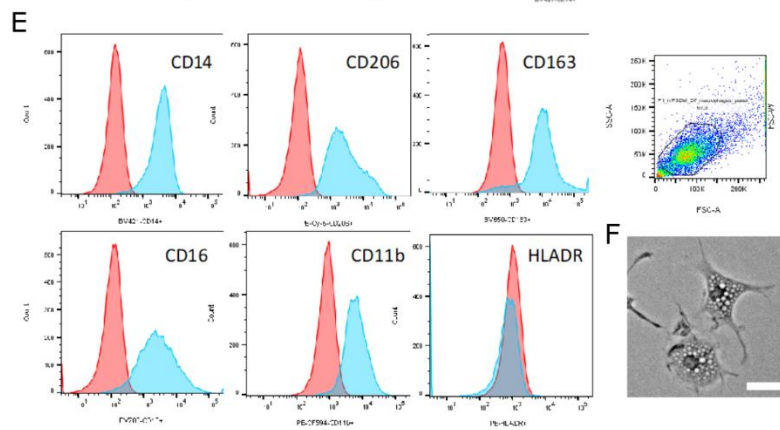
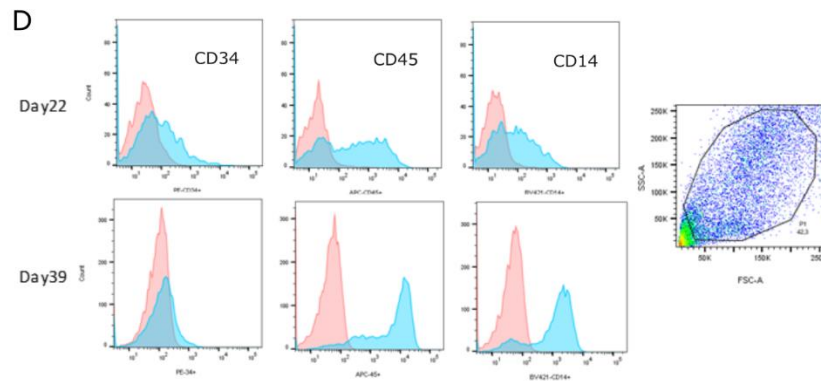
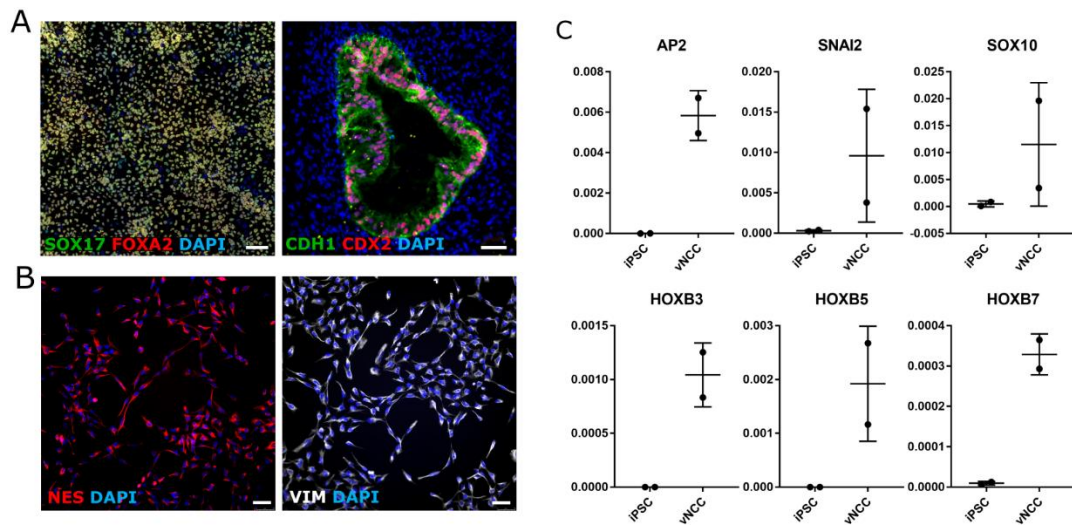


Figure S7. Characterization of hiPSC-derived human intestinal organoid, vagal neural crest, and macrophage, related to STAR Methods.

(A) Immunocytochemistry of markers for definitive endoderm (SOX17, FOXA2) and nucleus (DAPI) of endoderm monolayer during HIO derivation. Immunofluorescence of intestinal epithelial markers (CDH1, CDX2) in day 28 HIO. Scale bar=50µm.

(B) Immunocytochemistry for neural crest cell marker (NES, VIM) on the vagal neural crest cells. Scale bar=50µm.

(C) qPCR for genetic markers of neural crest (AP2, SNAI2, SOX10) and vagal fate (HOXB3, HOXB5, HOXB7) on the vagal neural crest cells (C1, C2). Normalized to GAPDH expression. hiPSC, n = 2, VNCC, n = 2. Biological replicates. Mean & s.d.

(D) Flow cytometry histograms showing staining (shaded blues) compared to the compensated unstained control (shaded red) for cell surface markers CD34, CD45 and CD14 on pre-macrophages released from adherent factory embryoid bodies (f-EB) harvested at day 22 and 39 since the beginning of the differentiation and the gating.

(E) Flow cytometry histograms showing cell surface markers CD14, CD206, CD163, CD16, CD11b and HLADR on macrophages differentiated from pre-macrophages and the gating.

(F) Bright field image of the differentiated macrophages. Scale bar=25µm.

Table S1. Oligonucleotides, related to Figure 2, 7, and S6.

Species	Target gene	Direction	Primer sequence
Human	<i>CCR2</i>	Forward	GGCATAGGGCAGTGAGAGTC
Human	<i>CCR2</i>	Reverse	TGTGAAAAAGGCTTCTGAACTTCT
Human	<i>CCR1</i>	Forward	TCCCTTGGAACCAGAGAGAAG
Human	<i>CCR1</i>	Reverse	ACCAAGGAGTACAGAGGGGG
Human	<i>CCR4</i>	Forward	AAAGCAAGCTGCTTCTGGTTG
Human	<i>CCR4</i>	Reverse	CTCCCCAAATGCCTTGATGC
Human	<i>CCR5</i>	Forward	ATCCAGTGAGAAAAGCCCGT
Human	<i>CCR5</i>	Reverse	TTCCACCCGGGGAGAGTTT
Human	<i>CCR6</i>	Forward	AAGAGAGGGCCCACGTGTAT
Human	<i>CCR6</i>	Reverse	ATTGATTCCCCGCTCATTGTG
Human	<i>CX3CR1</i>	Forward	TGGCCAAACACTGAGACCAA
Human	<i>CX3CR1</i>	Reverse	TGAAGGCCTCTAGTCGCTGT
Human	<i>CSF1R</i>	Forward	GTGGCTGTGAAGATGCTGAA
Human	<i>CSF1R</i>	Reverse	CCTTCCTTCGCAGAAAGTTG
Human	<i>FLT3</i>	Forward	CTCCAGGCGGCATCGC
Human	<i>FLT3</i>	Reverse	AAAACAACGAGCAGCGGCA
Human	<i>AP2</i>	Forward	ATGCTTTGGAAATTGACGGA
Human	<i>AP2</i>	Reverse	ATTGACCTACAGTGCCCAGC
Human	<i>SOX10</i>	Forward	AGCTCAGCAAGACGCTGG
Human	<i>SOX10</i>	Reverse	CTTTCTTGCTGCATACGG
Human	<i>SNAI2</i>	Forward	TGACCTGTCTGCAAATGCTC
Human	<i>SNAI2</i>	Reverse	CAGACCCTGGTTGCTTCAA
Human	<i>HOXB3</i>	Forward	CGTCATGAATGGGATCTGC
Human	<i>HOXB3</i>	Reverse	ATATTCACATCGAGCCCCAG
Human	<i>HOXB5</i>	Forward	GGAAGCTTCACATCAGCCAT
Human	<i>HOXB5</i>	Reverse	GGAACTCCTTTTCCAGCTCC
Human	<i>HOXB7</i>	Forward	AACTTCCGGATCTACCCCTG
Human	<i>HOXB7</i>	Reverse	CTTTCTCCAGCTCCAGGGTC
Human	<i>GAPDH</i>	Forward	GAAGGTGAAGGTCGGAGT
Human	<i>GAPDH</i>	Reverse	GAAGATGGTGATGGGATTTT
Human	<i>ENO1</i>	Forward	TCTCTTCACCTCAAAAGGTCTCT
Human	<i>ENO1</i>	Reverse	TGTGGGTTCTAAGGCTTACCC
Human	<i>TPI1</i>	Forward	GGACTCGGAGTAATCGCCTG
Human	<i>TPI1</i>	Reverse	GTACTTCCTGGGCCTGTTGG
Human	<i>LDHA</i>	Forward	CTGGCAAAGTGGATATCTTGAC
Human	<i>LDHA</i>	Reverse	ACTCCATACAGGCACACTGG
Human	<i>PGK1</i>	Forward	TTGACCGAATCACCGACCTC
Human	<i>PGK1</i>	Reverse	AGCAGCCTTAATCCTCTGGTT
Human	<i>PFKP</i>	Forward	AGGCAGTCATCGCCTTGCTAGA
Human	<i>PFKP</i>	Reverse	ATCGCCTTCTGCACATCCTGAG
Human	<i>HPRT1</i>	Forward	CCTGGCGTCGTGATTAGTGA
Human	<i>HPRT1</i>	Reverse	CGAGCAAGACGTTTCAGTCCT