Decoding human fetal liver haematopoiesis

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Definitive haematopoiesis in the fetal liver supports self-renewal and differentiation of haematopoietic stem cells and multipotent progenitors (HSC/MPPs) but remains poorly defined in humans. Here, using single-cell transcriptome profiling of approximately 140,000 liver and 74,000 skin, kidney and yolk sac cells, we identify the repertoire of human blood and immune cells during development. We infer differentiation trajectories from HSC/MPPs and evaluate the influence of the tissue microenvironment on blood and immune cell development. We reveal physiological erythropoiesis in fetal skin and the presence of mast cells, natural killer and innate lymphoid cell precursors in the yolk sac. We demonstrate a shift in the haemopoietic composition of fetal liver during gestation away from being predominantly erythroid, accompanied by a parallel change in differentiation potential of HSC/MPPs, which we functionally validate. Our integrated map of fetal liver haematopoiesis provides a blueprint for the study of paediatric blood and immune disorders, and a reference for harnessing the therapeutic potential of HSC/MPPs.

The blood and immune systems develop during early embryogenesis. Our understanding of this process derives from mouse and in vitro model systems, as human fetal tissue is scarce. Although haematopoietic development is conserved across vertebrates, there are notable differences between mouse and human. Comprehensive interrogation of human tissue to understand the molecular and cellular landscape of early haematopoiesis has implications beyond life in utero, as it provides a blueprint for understanding immunodeficiencies, childhood leukemias and anemias and generates insights into HSC/MPP propagation to inform stem cell technologies.

The earliest blood and immune cells originate outside the embryo, arising from the yolk sac between 2 and 3 weeks after conception. At 3–4 post-conception weeks (PCW), intra-embryonic progenitors from the aorta–gonad–mesonephros (AGM) develop. Yolk sac and AGM progenitors colonize fetal tissues such as the liver, which remains the major organ of haematopoiesis until the middle of the second trimester. Fetal bone marrow is colonized around 11 PCW and becomes the dominant site of haematopoiesis after 20 PCW in humans. Yolk sac–, AGM–, fetal liver– and bone marrow–derived immune cells seed peripheral tissues including non-lymphoid tissues (NLTs), where they undergo specific maturation programs that are both intrinsically determined and extrinsically nurtured by the tissue microenvironment.

In this study, we use single-cell transcriptomics to map the molecular states of human fetal liver cells between 7 and 17 PCW, when the liver is the predominant site of human fetal haematopoiesis. We integrate results from imaging mass cytometry, flow cytometry and cellular morphology to validate the transcriptome-based cellular profiles. We construct the functional organization of the developing immune network by comparative analysis of immune cells in fetal liver with those in yolk sac, skin and kidney as representative NLTs.

Single-cell transcriptome of fetal liver

To investigate blood and immune cell development in the fetal liver, we generated single-cell suspensions from embryonic and fetal livers between 7 and 17 PCW. We used fluorescence-activated cell sorting (FACS) to isolate CD45+ and CD45− cells using adjoining gates for comprehensive capture (Fig. 1a, Extended Data Fig. 9a) for single-cell RNA-seqencing (scRNA-seq) (both 10x Genomics platform and Smart-seq2) (Fig. 1 and Supplementary Table 1). To enable parallel evaluation of blood and immune cell topography in NLT and the yolk sac...
Sac during early development (Fig. 1a), we profiled skin, kidney and yolk sac cells using isolation by FACS and the 10x Genomics platform.

In total, 138,575 (n = 14) (an additional 1,206,120 cells were profiled using Smart-seq2), 54,690 (n = 7) skin, 9,643 kidney (n = 3) and 10,071 yolk sac (n = 3) cells passed quality control and doublet exclusion (Extended Data Fig. 1a, b, Supplementary Table 2). We performed graph-based Louvain clustering and identified differentially expressed genes (DEGs) to annotate cell clusters. To minimize technical batch effects while preserving biological variation due to gestational stage, we divided liver samples into four gestational-stage categories and performed data integration between samples using Harmony (Extended Data Fig. 1c–e).

We identified 27 major cell states in the fetal liver (Fig. 1b, Extended Data Fig. 1f). VCAM1\(^+\) erythroblastic island (EI) macrophages were validated as a distinct cell state owing to their interactions with erythroid cells (Extended Data Fig. 4a–f). We applied a descriptive nomenclature on the basis of gene-expression profiles. All cell states were found throughout the developmental period studied, but the frequency varied according to gestation stage (Fig. 1c, Extended Data Fig. 1e). Neutrophils, basophils and eosinophils were not detected, consistent with reports of granulocytes emerging during fetal bone marrow hematopoiesis. Samples from early stages exhibited erythroid lineage bias, and lymphoid and myeloid lineages were represented at later stages, as previously shown (Fig. 1c, Extended Data Fig. 1g).

Our fetal liver dataset can be explored using an interactive web portal at https://developmentcellatlas.ncl.ac.uk/datasets/bca_liver/. We provide comprehensive expression profiles of genes that are known to cause primary immunodeficiencies to aid future molecular phenotyping of these disorders (Extended Data Fig. 8).

Validation of genes and cell states

We manually selected 48 genes from the 4,471 DEGs between all clusters (log(fold change) > 0.5) (Fig. 2a). The predictive power of these 48 genes to determine cell states by the Random Forest classifier was 89% on average for precision and recall (Extended Data Fig. 2a). We designed a FACS panel for prospective cell isolation using genes that encode cell surface proteins (Fig. 2a, Extended Data Fig. 9b), which enabled validation of 19 cell types by mini bulk transcriptome profiling using Smart-seq2 (Extended Data Fig. 2b–d); 6 of the 19 cell types were also validated by scRNA-seq. Cytospins from FACS-isolated cells were morphologically consistent with their designated cell type, including hypogranularity of embryonic or fetal mast cells and the resemblance of early erythroid cells to previously reported ‘early erythroid progenitors’ (Fig. 2b).

Next, we evaluated the spatial distribution of erythroid, mast cell, myeloid and lymphoid lineages using imaging mass cytometry (Extended Data Fig. 2e). The liver architecture evolved considerably between 8 and 15 PCW. Organization of hepatocyte aggregates increased, although hepatic lobules around a central vein and portal triad are not clearly visible. Haematopoietic islands were present in sinusoids and surrounding hepatocyte aggregates. Sinusoidal CD68\(^+\) macrophages were surrounded by glycoporphrin A (GPYA)\(^+\) erythroblast cells (Extended Data Fig. 2e). CD11c\(^+\) dendritic cells (DCs) and CD79a\(^+\)CD20\(^-\) cells from the B cell lineage were sparsely distributed (Extended Data Fig. 2e). The proportions of these cells approximated our scRNA-seq profile for haematopoietic cells but not that for hepatocytes (Extended Data Fig. 2e, Fig. 1b), in keeping with the fragility of hepatocytes following ex vivo isolation and their high expression of mitochondrial genes. By validating our single-cell transcriptome dataset with multiple modalities, we provide an integrated map of haematopoietic cells in the fetal liver.

Fetal liver and NLT haematopoiesis

Next, we inferred trajectories of haematopoietic development. By force-directed graph (FDG) analysis, we identified three connections to a central HSC/MPP node featuring erythroid–megakaryocyte–mast cells, B cells and innate or T-lymphoid cells and myeloid cells (Fig. 3a and Supplementary Video 1). Partition-based approximate graph abstraction (PAGA) also supported the presence of a shared megakaryocyte–erythroid–mast cell progenitor (MEMP) downstream of HSC/MPPs (Extended Data Fig. 3a). Genes that were dynamically modulated in the specification of erythroid, megakaryocyte and mast cell lineages were distinct: TAL1 and KLF1 in the erythroid lineage, F11R, PBX1 and MEIS1 in the megakaryocyte lineage and HES1 in mast cell differentiation (Extended Data Fig. 3b). We investigated supporting factors for fetal liver erythropoiesis using CellPhoneDB to predict specific or enriched receptor–ligand interactions between erythroblasts and VCAM1\(^+\) El macrophages (Extended Data Fig. 4a). We identified statistically significant interactions for VCAM1, ITGB1, ITGA4, SIGLEC1, IACM4 and SPN, which encode molecules that are known to be important in haematopoiesis (Extended Data Fig. 4a). The presence of VCAM1 on El macrophages and ITGA4 on early to-mid erythroid cells was confirmed by immunohistochemical analysis on serial fetal liver sections (Extended Data Fig. 4b). Interaction of VCAM1\(^+\) El macrophages with erythroblasts was also observed using
imaging flow cytometry analysis (Extended Data Fig. 4d) and may explain the combined erythroblast and macrophage transcriptome of VCAM1⁺ EI macrophages (Fig. 2a), which has also been described in mouse central EI macrophages (Extended Data Fig. 4g).²⁰

Comparing across haematopoietic tissues, mast cells were also present in the yolk sac (Extended Data Fig. 1b). Erythroblasts show expression of haemoglobin genes and a temporal shift from expression of HBZ and HBE1 to expression of fetal haemoglobin subunits (HBA1 and HBG2) between yolk sac and liver (Fig. 3b).

Fig. 2 | Multi-modal and spatial validation of cell types. a, Median-scaled ln-normalized gene expression of 48 selected DEGs for the liver cell states from Fig. 1b. Asterisks indicate markers used for isolation of cells by FACS. Gene-expression frequency (percentage of cells within each cell type expressing the gene) is indicated by spot size and expression level is indicated by colour intensity. Mono, monocyte; pDC, precursor. b, Representative Giemsa-stained cytospins showing morphology of populations isolated by FACS on the basis of DEGs marked with asterisks in a. Scale bars, 10 µm.

Megakaryocytes, erythroblasts, mast cells and MEMPs were present in NLTs, but HSC/MPPs were absent (Extended Data Figs. 1a, 3c–e). We compared the highly and differentially expressed genes of corresponding cell types in fetal liver, skin and kidney (Extended Data Fig. 3d). Mast cells, megakaryocytes and cells of the erythroid lineages showed high connectivity (PAGA scores) across all four tissues (Extended Data Fig. 3e). This high connectivity may be a result of local maturation of progenitors in NLT or influx of cells at various differentiation stages. Erythroid cells were absent in kidney, suggesting restricted

Fig. 3 | Fetal liver and NLT haematopoiesis. a, FDG visualization of all haematopoietic cells from Fig. 1b. b, Dot plot showing the median-scaled ln-normalized expression of globin genes encoding haemoglobin subunits: HBZ and HBE1 (Gower 1), HBE1 and HBA1 (Gower 2) and HBA1 and HBG2 (fetal; HbF) in liver, skin and yolk sac (YS) erythroid lineages (MEMP and early, mid and late erythroblasts). Gene-expression frequency (percentage of cells within each cell type expressing the gene) is indicated by spot size and expression level is indicated by colour intensity.
changes over four developmental stages. b, FDG visualization of fetal liver and corresponding skin, kidney and yolk sac lymphoid cells.

**Lymphoid lineages in fetal liver and NLT**

Previous studies have reported the presence of T and B lymphocytes, natural killer (NK) cells, and innate lymphoid cells (ILCs) in the human fetal liver. In our analysis, we observed two lymphoid branches: an NK–T–ILC lineage and a B lineage (Fig. 4a, Extended Data Fig. 5a). The early lymphoid/T lymphocyte cluster varied by gestational stage, with cells expressing GATA3, KLRB1, CD3D, CD7 and JCHAIN at 7–8 PCW before T cells emerged from the thymus (Fig. 4a, Extended Data Fig. 5b, c). Early in gestation, this cluster may contain fetal liver early thymocyte progenitors, which are capable of generating γδ T cells when co-cultured with thymic epithelial cells.35–37 At the 12–14 PCW stage, cells expressed TRDC and TRAC, but not GZMB or PRF1, the cytolytic granular products characteristic of mature CD8+ T cells. TRDC expression was absent at the 15–17 PCW stage (Extended Data Fig. 5b–d). These findings are consistent with seeding of fetal liver by γδ T cells and αβ T cells sequentially following their exit from thymus after 12 PCW37 and are consistent with previous reports of T cell identification only being possible after 18 PCW.36,38

**Tissue signatures in myeloid cells**

Fate-mapping studies in mice have demonstrated that tissue macrophages are seeded by yolk sac and fetal liver progenitors, whereas DCs originate from bone marrow-derived HSC/MPPs through a monocyte-independent lineage.35 We observed myeloid progenitors, monocytes, macrophages and DC1 and DC2 clusters in fetal liver and NLT as early as 7 PCW (Figs. 1b, 5a, Extended Data Figs. 1a, 6a).

Myeloid lineages stemmed from HSC/MPP via three intermediates: a neutrophil–myeloid progenitor expressing CD34, SPINK2, AZU1, PRTN3, ELANE, MPO and LYZ, monocyte precursors and DC

Fig. 4 | Lymphoid lineages in fetal liver and NLT. a, FDG visualization of fetal liver HSC/MPP and lymphoid cell types from Fig. 1b showing differentiation of the MEMP lineage at specific sites (Extended Data Fig. 1a). Immunohistochemical analysis of serial skin sections showed nucleated GYPA+ cells inside and outside CD34+ blood vessels, in keeping with local differentiation of MEMPs (Extended Data Fig. 3f). Light-sheet fluorescence microscopy supported this finding (Extended Data Fig. 3g, Supplementary Video 2). The proliferative capacity of MEMP in NLT was confirmed by expression of MKI67 and cell cycle genes (Extended Data Fig. 3h). Skin MEMPs expressed some early erythroblast genes including MYL4 (Extended Data Fig. 3d), suggesting that these may act as erythroid progenitors in the skin.32 These findings demonstrate that during early development, skin in the physiological state can contribute to erythropoiesis and supplement the erythroid output of fetal liver.

**Tissue signatures in developing myeloid cells.** a, FDG visualization of HSC/MPP, myeloid progenitors, monocytes and macrophages from fetal liver, decidua/placenta and yolk sac. b, PAGA connectivity scores of the populations shown in a.
megakaryocyte. d, Percentage of colonies generated by single HSC pool gated cells containing erythroid cells (defined as the sum of ery, ery–meg, ery–meg–my, ery–ery, ery–NK and ery–NK–my colonies shown in c); ***P < 0.001. e, Percentage of colonies containing B cells following culture in conditions optimized for B and NK cells from 10 colonies from the HSC pool gate. **P < 0.01. f, Percentage of cells in the G0 phase of the cell cycle assessed using Ki67 and DAPI flow cytometry analysis (P = 0.0136). Data are mean ± s.d. g, In-normalized median expression of selected genes in yolk sac progenitors, cord blood HSCs and adult bone marrow (BM) HSCs with significant differential expression compared to fetal liver HSC/MPP, visualized by violin plots (****P < 0.0001).

**HSC/MPP differentiation potential by age**

Our observation of a HSC/MPP cell state from which the earliest lineage-committed cells radiate is in line with recent observations from scRNA-seq analysis in postnatal mice and humans\(^{36,43,44}\) (Fig. 6a). At the base of this cluster by FGD visualization was a population expressing CLEC9A, HLA-DRA and the highest levels of primitive genes including MLLT3, consistent with a multipotent long-term repopulating (LT)-HSC population\(^{36}\) (Fig. 6a, b). Using DEGs and supervised analysis, HSC/MPP clusters with gene expression intermediate between LT-HSCs and early progenitors similar to human lymphoid-primed multilymphoid progenitor (MLP) gate also classified as HSC/MPP (Extended Data Fig. 7b). A support vector machine trained on the fetal liver dataset identified S100A8/A9, FCGR1A/2A, S100A8/A9, FCGR1A/2A, BATF3 and ID2, consistent with a multipotent long-term repopulating (LT)-HSC population\(^{36}\) (Fig. 6a, b). Using DEGs and supervised analysis, HSC/MPP clusters with gene expression intermediate between LT-HSCs and early progenitors similar to human lymphoid-primed short-term (ST)-HSCs\(^{46-48}\), the mouse erythroid-biased MMP2 and myeloid-biased MMP5\(^{46,47}\) were identifiable, demonstrating early transcriptome priming along all differentiation branches within the MPP pool (Fig. 6a, b).

We hypothesized that the cellular composition of the developing fetal liver resulted from local modulation of HSC/MPP potential. To test this, we used FACS to isolate single cells from the CD34—CD38\(^+\), CD34—CD38—CD45RA— and CD34—CD38—CD45RA— FACS gates and profiled them by plate-based single-cell transcriptomics (Smart-seq2) and single-cell clonal differentiation assays\(^{45,48}\) (Extended Data Fig. 7a). A support vector machine trained on the fetal liver dataset identified enrichment of HSC/MPPs (to about 85%) in the CD34—CD38— gate, with the majority of cells in the CD34—CD38—CD45RA— multilymphoid progenitor (MLP) gate also classified as HSC/MPP (Extended Data Fig. 7b). This is in agreement with reported transcriptional similarity between HSC/MPP and MLP\(^{49}\) and our identification of lymphoid priming in the HSC/MPP compartment. Single-cell culture from CD34—CD38—CD45RA— HSC/MPPs yielded both uni- and multipotential colonies (Fig. 6c–e, Extended Data Figs. 7c–g, 9c, d). There was a significant reduction in trilineage colonies with gestational age (Extended Data Fig. 7f). Colonies containing erythroid cells significantly decreased in number, whereas those containing NK cells and B cells increased with gestational age (Fig. 6c–e, Extended Data Fig. 7c–g).

HSC/MPP from embryonic livers at less than 9 PCW generated almost

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**Fig. 6** HSC/MPP differentiation potential by gestation. a, FGD visualization of liver HSC/MPP and early haematopoietic progenitor populations from Fig. 1b. b, Violin plots showing ln-normalized median gene expression of statistically significant, dynamically variable genes that are up- or downregulated during HSC/MPP transition to neutrophil–myeloid progenitors, MEMP and pre pro-B cells from fetal liver. NS, not significant. H/M, HSC/MPP. c, Stacked bar plot of all different types of colonies generated by single ‘HSC pool’ gated cells in an assay with MS5 stromal cells. *P < 0.05, **P < 0.001; individual samples shown in Extended Data Fig. 7c. The colour of the bar corresponds to the type of colony tested compared with all others. My, myeloid; ery, erythroid, meg.
no B cells (Fig. 6e), in keeping with the paucity of B cells at this stage (Figs. 1c, 4a, Extended Data Fig. 1g). These findings support the hypothesis of differential HSC/MPP intrinsic potential according to gestational stage and mirror our observation of early erythroid predominance and greater lymphoid representation at later stages (Figs. 1c, 4a).

Comparing HSC/MPPs and early progenitors across haematopoietic tissues during development, higher MXI67 expression and cell cycle staining suggest enhanced proliferative potential of yolk sac and fetal liver progenitors and HSC/MPPs (Extended Data Fig. 7h). The fraction of fetal liver HSC/MPPs in G0 increased with gestational age (Fig. 6f, Extended Data Fig. 7i), indicating a progressive shift to quiescence during fetal life. Fetal liver HSC/MPPs showed higher expression of genes encoding a heat shock protein (HSPA1A), potentially for maintenance of genome and proteome integrity, and lower levels of MHC-I (HLA-B), suggesting reduced antigen-presenting potential compared with cord blood and adult bone marrow HSC/MPPs (Fig. 6g).

Collectively, our findings demonstrate that intrinsic changes in HSC/MPP numbers, proliferation and differentiation potential occur over the first and second developmental trimesters. These changes are likely to be pivotal for fetal liver haematopoiesis to adapt to the needs of the developing fetus; first the establishment of an effective oxygen transport system and subsequently the development of a complete blood and immune system.

Discussion

Development of the human immune system in utero has remained poorly understood. Using single-cell transcriptome profiling, we have resolved the cellular heterogeneity and abstract dynamic temporal information on blood and immune development in fetal liver, yolk sac, skin and kidney. Large-scale scRNA-seq studies during human development must account for technical batch effects without compromising detection of biological variations over gestation. Our approach highlights key insights: physiological erythropoiesis in fetal skin, establishment of a DC network as early as 7 PCW, potential dual myeloid establishment of a DC network as early as 7 PCW, potential dual myeloid

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METHODS
The experiments were not randomized. Unless otherwise stated, the investigators were not blinded to allocation during experiments and outcome assessment.

Tissue acquisition. Human developmental tissues were obtained from the MRC–Wellcome Trust-funded Human Developmental Biology Resource (HDBR; http://www.hdbr.org) with appropriate written consent and approval from the Newcastle and North Tyneside NHS Health Authority Joint Ethics Committee (08/H1006/115). HDBR is regulated by the UK Human Tissue Authority (HTA; www.hta.government.uk) and operates in accordance with the relevant HTA Codes of Practice. Embryos and fetal specimens used for light-sheet fluorescence microscopy were obtained with written informed consent from the parents (Gynaecology Hospital Jeanne de Flandres, Lille, France) with the approval of the local ethics committee (protocol no. PFS16-002). Tissues were made available in accordance with the French bylaw (Good practice concerning the conservation, transformation and transportation of human tissue to be used therapeutically, published on December 29, 1998). Permission to use human tissues was obtained from the French agency for biomedical research (Agence de la Biomedecine, Saint-Denis La Plaine, France). No statistical methods were used to predetermine sample size.

Tissue processing. All tissues were processed immediately after isolation using the same protocol. Tissue was transferred to a sterile 10 mm2 tissue culture dish and cut into <1 mm3 segments before being transferred to a 50-ml conical tube. Yolk sac content was aspirated for analysis before yolk sac digestion. Tissue was digested with 1.6 mg ml−1 collagenase type IV (Worthington) in RPMI (Sigma-Alrich) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco), 100 U ml−1 penicillin (Sigma-Alrich), 0.1 mg ml−1 streptomycin (Sigma-Alrich), and 10% (v/v) heat-inactivated fetal calf serum for 30 min at 37°C with intermittent shaking. Digested tissue was passed through a 100-μm filter, and cells were collected by centrifugation (500g for 5 min at 4°C). Cells were treated with 1 × RBC lysis buffer (eBioscience) for 5 min at room temperature and washed once with flow buffer (PBS containing 5% (v/v) FBS and 2 mM EDTA) before counting.

Fetal developmental stage assignment and chromosomal assessment. Embryos up to 8 PCW were staged using the Carnegie staging method44. After 8 PCW, developmental age was estimated from measurements of foot length and heel-to-knee length and compared against a standard growth chart45. A piece of skin, or where this was not possible, chorionic villi tissue was collected from every sample for quantitative PCR analysis using markers for the sex chromosomes and autosomes 13, 15, 16, 18, 21, 22, which are the most commonly seen chromosomal abnormalities. All samples were karyotypically normal.

Flow cytometry and FACS for scRNA-seq. Antibody panels were designed to allow enrichment of cell fractions for sequencing and cell type validation. Antibodies used for FACS isolation are listed in Supplementary Table 14. An antibody cocktail was prepared fresh by adding 3 μl of each antibody in 50 μl Brilliant Stain Buffer (BD Biosciences) per tube. Cells (≤1 × 106) were resuspended in 50–200 μl of cell-type equivalent volumes of wash buffer and stained with antibodies and subsequently run on a BD FACSAria Fusion instrument using DIVA v.8, and data were analysed using Flowjo (v.10.4.1, BD Biosciences). Cells were gated to exclude dead cells and doublets, and then isolated for scRNA-seq analysis (10x or Smart-seq2). For 10x sequencing, cells were sorted into chilled FACS tubes coated with FBS and prefilled with 500 μl sterile PBS. For Smart-seq2, single cells were index-sorted into 96-well LoBind plates containing 10 μl per well lysis buffer for Smart-seq2 (Supplementary Table 12). Analysis of single-cell-derived colonies was performed as described45. In brief, colonies were collected into 96 U-bottom plates using a plate filter to prevent carryover of MS5 cells. Cells were stained with 50 μl per well of antibody cocktail (Supplementary Table 16, 17 for antibody details), incubated for 20 min in the dark at room temperature and then washed with 100 μl per well of PBS + 3% FBS. The type (lineage composition) and the size of the colonies formed were assessed by high-throughput flow cytometry (BD FACS Symphony). Colony output was determined using the gating strategy shown in Extended Data Fig. 9c. A single cell was defined as giving rise to a colony if the sum of colonies identified in each antibody-gated cell was >30 cells. Overt doublets present as having an increased area and a lower aspect ratio value and were also gated as such. In all high-throughput screening flow cytometry data were recorded in a blinded way, and correlation between the colony phenotype and originating population was only performed at the final stage. Two-tailed Fisher’s exact tests, performed in Prism (v8.1.2, GraphPad Software), were applied to the numbers of colonies of each type by stage to determine statistical significance in doublet rates. For 10x sequencing, cells were sorted into chilled FACS tubes coated with FBS and prefilled with 100 μl sterile PBS. For Smart-seq2, single cells were index-sorted into 96-well LoBind plates containing 10 μl per well lysis buffer for Smart-seq2 (Supplementary Table 12). Analysis of single-cell-derived colonies was performed as described45. In brief, colonies were collected into 96 U-bottom plates using a plate filter to prevent carryover of MS5 cells. Cells were stained with 50 μl per well of antibody cocktail (Supplementary Table 16, 17 for antibody details), incubated for 20 min in the dark at room temperature and then washed with 100 μl per well of PBS + 3% FBS. The type (lineage composition) and the size of the colonies formed were assessed by high-throughput flow cytometry (BD FACS Symphony). Colony output was determined using the gating strategy shown in Extended Data Fig. 9c. A single cell was defined as giving rise to a colony if the sum of colonies identified in each antibody-gated cell was >30 cells. Overt doublets present as having an increased area and a lower aspect ratio value and were also gated as such. In all high-throughput screening flow cytometry data were recorded in a blinded way, and correlation between the colony phenotype and originating population was only performed at the final stage. Two-tailed Fisher’s exact tests, performed in Prism (v8.1.2, GraphPad Software), were applied to the numbers of colonies of each type by stage to determine statistical significance in doublet rates.

ImageStream analysis of fetal liver cell suspensions. Frozen fetal liver cells were thawed and stained with the antibody cocktail (see Supplementary Table 19 for antibody details). Cells were washed twice with cell type equivalent volumes of wash buffer and resuspended at the same cell density that was used for cell-sorting experiments (1 × 106 cells per ml). DAPI (Sigma-Alrich) was added to a final concentration of 3 μM immediately before acquisition to identify and exclude dead cells from the experiment. Samples were acquired on a fully calibrated ImageStream X MKII system (Luminex Corporation) using 488 nm, 561 nm, 405 nm and 642 nm excitation wavelengths and the 60× magnification collection optic. Laser powers were set in order to maximize signal resolution but minimize any saturation of the CCD camera with bright-field images collected in channels 1 and 9. A minimum of 50,000 cell events were collected per sample. In order to calculate spectral compensation, single-stained antibody capture beads were acquired with the bright-field illumination turned off. Spectral compensation and data analysis were performed using the IDEAS analysis software (v6.2.64, Luminex Corp). In brief, dead cells were first excluded based on DAPI positivity. Overt doublets and debris were excluded from the live cell population using the aspect ratio and area of the bright-field image in channel 1. Single cells had an intermediate area value and an aspect ratio between 1 and 0.6 were gated as such. Overt doublets present as having an increased area and a lower aspect ratio value and were also gated as such. In all cases, the position and boundaries of a gate were checked for appropriateness using the underlying imagery. The key cell types within the sample were then identified and gated using the total integrated (post-compensation) fluorescence signals from each labelled antibody in the panel in an analogous fashion to conventional flow cytometry data. Each major cell type was then interrogated using the associated multi-spectral images for true single-cell identity or for the presence of ‘pernicious doublets’ (cells with either significant debris attached or large cells with much smaller cells attached). The same phenotypic analysis was extended to the cells in the overt doublet gate. Further analysis was performed in Flowjo (FlowJo, LLC) using software supplied post hoc by scRNAseq (supplementary software). Analysis was performed from the distinct doublet gate within the doublet gate to determine statistical significance in doublet ratios across fetal liver cell types.
Library preparation and sequencing. For the droplet-encapsulation scRNA-seq experiments, 7,000 live, single, CD45− or CD45+ FACS-isolated cells were loaded onto each channel of a Chromium chip before encapsulation on the Chromium Controller (10x Genomics). Single-cell sequencing libraries were generated using the Single Cell 3′ v2 and V(DJ) Reagent Kits (for T cell receptor repertoire analysis) as per the manufacturer's protocol. Libraries were sequenced using an Illumina HiSeq 4000 using v4 SBS chemistry to achieve a minimum depth of 50,000 raw reads per cell. The libraries were sequenced using the following parameters: Reads1: 26 cycles; I7: 8 cycles; I5: 0 cycles; Reads2: 98 cycles to generate 75-bp paired-end reads.

For the plate-based scRNA-seq experiments, a slightly modified Smart-seq2 protocol was used as previously described57. After CDNA generation, libraries were prepared (384 cells per library) using the Illumina Nextera XT index. Index v2 sets A, B, C and D were used to pool each sample before multiplexing. Each library was sequenced to achieve a minimum depth of one-to-two-million raw reads per cell using an Illumina HiSeq 4000 using v4 SBS chemistry to generate 75-bp paired-end reads.

For the mini bulk RNA-seq experiments, each cell lysate was transferred into a 96-well low-binding plate (Eppendorf) then processed using the same modified Smart-seq2 protocol as described above. After CDNA generation, libraries were prepared using the Illumina NexteraXT kit with Index v2 set A to barcode each mini bulk library before multiplexing. All libraries were sequenced on one lane of an Illumina HiSeq 4000 using v4 SBS chemistry to generate 75-bp paired-end reads and aiming to achieve a minimum depth of 10 million reads per library.

Immunohistochemistry. Formalin-fixed, paraffin-embedded blocks of fetal livers aged 6, 12, 25, 50, 75, and 100 were obtained from the HDBR. Each was sectioned at 4-μm thickness onto APEX-coated slides. Sections were dewaxed for 5 min in Xylene (Fisher Chemical) then rehydrated through graded ethanol (99%, 95% and 70%; Fisher Chemical) and washed in running water. Sections were treated with hydrogen peroxide block (1% v/v in water; Sigma) and then slides placed in TBS pH 7.6 for 5 min before staining. Staining was done using the Vector ImmPRESS Kit (Vector Laboratories). Sections were then blocked sequentially with 2.5% normal horse serum, avidin (Vector Laboratories) and then biotin (Vector Laboratories) for 10 min each and then washed in TBS pH 7.6. Sections were incubated for 60 min with primary antibody diluted in TBS pH 7.6 (see Supplementary Table 20 for antibody details). Slides were washed twice in TBS pH 7.6 for 5 min each before incubation for 30 min with the secondary antibody supplied with the kit. Slides were washed twice in TBS pH 7.6 for 5 min each, and developed using peroxidase chromogen DAB. Sections were counterstained in Mayer's haematoxylin for 30 s, washed and put in Scott's tap water for 30 s. Slides were dehydrated through graded ethanol (70% to 99%) and then placed in xylene before being mounted in DPX (Sigma-Aldrich). Sections were imaged on a Nikon Eclipse 80i microscope using NIS-Elements Fv1.

Alignment, quantification and quality control of scRNA-seq data. Droplet-based (10x) sequencing data were aligned and quantified using the Cell Ranger Single-Cell Software Suite (v.2.0.2, 10x Genomics) using the GRCh38 human reference genome (official Cell Ranger reference, v1.2.0). Smart-seq2 sequencing data were aligned with STAR (v.2.5.1b), using the STAR index and annotation from the same reference as the 10x data. Gene-specific read counts were calculated using htseq-count (v0.10.0). Cells with fewer than 200 detected genes and for which the total mitochondrial gene expression exceeded 20% were removed. Genes that were expressed in fewer than 3 cells were also removed. We detected on average ~3,000 genes per cell with the 10x Genomics platform and ~6,000 genes with the Smart-seq2 protocol.

Doublet detection. Doublets were detected with an approach adapted from a previous study38. In the first step of the process, each 10x lane was processed independently. Scramble was run, obtaining per-cell doublet scores. The standard Seurat-inspired Scanny processing pipeline was performed up to the clustering stage, using default parameters. Each cluster was subsequently separately clustered again, yielding an over-clustered manifold, and each of the resulting clusters had its Scramble scores replaced by the median of the observed values. The resulting scores were assessed for statistical significance, with P values computed using a right-tailed test from a normal distribution centred on the score median and a median absolute deviation (MAD)–derived standard deviation estimate. The MAD was computed from above–median values to circumvent zero truncation. The P values were corrected for false discovery rate with the Benjamini–Hochberg procedure, and a significance threshold of 0.1 was imposed. In the second step of the process, all 10x lanes for a single tissue were pooled together and the Seurat-inspired Scanny processing was repeated, with the addition of Harmony, with a theta of 3, for batch correction between the lanes before the neighbour graph identification step. The joint manifold was clustered, and the frequency of identified doublets was computed. The same statistical framework as in the first step was used to identify clusters significantly enriched in doublets, which were subsequently flagged as doublets in their entirety and removed.

Clustering and annotation. Downstream analysis included data normalization (NormalizeData, LogNormalize method, scaling factor 10,000), data feature scaling (ScaleData), variable gene detection (FindVariableGenes), PCA (RunPCA, from variable genes) and Louvain graph-based clustering (FindClusters, data dimensionality reduction using PCA, clustering resolution (res.30)) performed using the R package Seurat (v2.3.4). Cluster identity was assigned by manual annotation using known marker genes and computed DEGs using the FindAllMarkers function in the Seurat package (one-tailed Wilcoxon rank sum test; P values adjusted for multiple testing using the Benferroni correction; Supplementary Table 3). For computing DEGs, all genes were probed were they expressed in at least 25% of cells in either of the two populations compared and the expression difference on a natural log scale was at least 0.25. Manual annotation was performed iteratively, which included validating proposed cell labels with known markers and further investigating clusters for which the gene signatures indicated additional diversity.

Numbers of each cell type per sample, annotations per cell, and nGene and nUMI per cell type are reported in Supplementary Tables 4, 5, 8 and 9, respectively.

Clustering and cell type assignment for fetal liver data was assessed using two additional clustering methods (not shown): agglomerative clustering (with Ward linkage and Euclidean affinity) and Gaussian mixture (AgglomerativeClustering class from the cluster module and GaussianMixture from the mixture module in sklearn v0.19.1 Pyton 3.6.3). Consensus agreement between the three clustering methods was measured by Rand index and adjusted mutual information implemented in the metrics module in the sklearn package. The Rand index scores were 0.48 and 0.085 for agglomerative and Gaussian mixture clustering methods, respectively.

After annotation was completed, a cell type classifier was built by training a support vector machine (SVM) on labelled fetal liver scRNA-seq data with grid search for parameter optimization based on training data. Seventy per cent of the data was used for training and the other 30% for test. The SVM was previously compared in terms of accuracy and recall with a random forest and logistic regression classifiers trained on the same data. Out of the three classifiers, the SVM was chosen because it showed a mean accuracy and weighted mean recall of 95%. Random forest showed 89% for both precision and recall (Supplementary Table 11). The SVM classifier was used for automatic annotation of the Smart-seq2 and mini bulk RNA sequencing datasets to enable identification of biologically meaningful clusters and DEG computation.

Data generated from fetal skin, kidney and yolk sac were pre-processed, normalized, clustered and manually annotated in parallel with, and using the same pipeline as, the liver data. Annotation by cell type for skin and kidney, and yolk sac are reported in Supplementary Tables 6 and 7, respectively. Skin and kidney data were combined using the MergeSeurat function. Clusters characterized by expression of known immune markers were extracted from the NLT dataset for subsequent comparative analysis with liver-derived immune populations. Human cord blood and adult bone marrow datasets were downloaded from the Human Cell Atlas data portal (https://data.humancellatlas.org/). These were processed using the same approach as described above, followed by manual annotation. Decidua and placental data from ref. 52 were downloaded from ArrayExpress record E-MTAB-6701.

Data integration. We used Harmony data integration to correct for batch effect between sample identities. The average kBT rejection rate statistically significantly improved from 0.735 to 0.471 (Supplementary Table 13) following Harmony data integration (P = 3.83 × 10−6 in Kolmogorov–Smirnov test and P = 8.8 × 10−4 in Wilcoxon signed-rank test). The manifold was subjected to re-clustering using Harmony adjusted principal components (PCs) with parameters as mentioned above in 'Clustering and annotation'. Cell-type classifications were then ascertained through re-annotation of the clusters derived from Harmony-adjusted PCs to produce the final annotation.

Changes in cell proportions over development. Comparison of cell proportions across gestational stages was assessed by modelling cell number data with negative binomial regression based on Poisson-gamma mixture distribution. Cell numbers were corrected for CD45− CD45+ FACS-sorted ratio (Supplementary Table 1) before applying negative binomial regression modelling. Modelling was achieved using the glm.nb function in the R MASS package. Modelled cell number data were studied for regression coefficient significance (variable coefficient ≤ 0.05) to the response variable of gestational age with the corresponding P values taken (Supplementary Table 10).

Dimensionality reduction and trajectory analysis. Dimensionality reduction methods included t-distributed stochastic neighbor embedding (t-SNE), UMAP (Python UMAP package, five nearest neighbours, correlation metric, minimum distance 0.3, computed from the first 20 PCs), FDG (ForceAtlas2 class from fa2 Python Package, Barnes–Hut implementation for faster computation with theta 0.8, 2,000
iterations) and PAGA (paga in scancy Python package v1.2.2). Development trajectories were inferred by comparing FDG, PAGA and diffusion-map plots. Inferred trajectory analysis included computing diffusion map (scanny tl.diffmap with 20 components), pseudotime (scanny tl.dpt setting the earliest known cell type as root) and variable genes across pseudotime. The order of cells in pseudotime was statistically significant using Kruskal–Wallis test (P < 1 × 10^{-3}).

Comparisons of trajectories across stage were performed by subsetting liver dataset by stage using the SubsetData function, computing dimensional reduction coordinates, batch correcting by sample using Harmony and plotting PAGA and FDG by stage. Cell-type comparisons across tissue involved subsetting for cell types of interest using the SubsetData function, merging cross-tissue datasets using the MergeSeurat function and processing data using the same approach as for the liver and NLT datasets. Harmony batch correction was then performed by tissue type, with results presented as combined UMAPs, FDGs and PAGA score heatmaps.

Dynamically expressed genes across pseudotime. Genes that vary across pseudotime were calculated using the DifferentialGeneTest function in Monocle 2 in R (v.2.6.4) and a cut-off of adjusted P value < 0.001 was applied. This was applied on the entire pseudotime range and also on the pseudotime intervals specific to each cell type in order to avoid limitation to the genes characterized by monotonic changes across the inferred trajectory. Expression of pseudotime-variable genes were min – max normalized before visualization and annotated based on the involvement of each gene in relevant cell-specific functional modules or hallmark functional pathways from MSigDB v6.2, a curated molecular signature database. Peak expression for each gene over pseudotime was calculated and grouped into ‘early,’ ‘mid’ or ‘late’ categories. For visualization purposes, the resulting genes lists were min-max normalized and ordered on the reads from those present in the most selected functional pathways to least, as well as ensuring coverage across pseudotime. These genes were manually compared against current literature to determine if they have known functional or cell type associations. The top 20 – 25 genes in each list were displayed using the ggplot2 package. Transcription factors were marked within the dataset based on AnimalTFDB transcription factor prediction database.

The full pseudotime gene list is available in the interactive files accompanying diffusion maps.

Mouse data generated previously, which were used for comparison of erythroid–associated genes expressed in mouse Kupffer cells versus EI macrophages shown in Extended Data Fig. 4g, are available at the Gene Expression Omnibus with accession code GSE127980.

Visualization by animated FDG representation. The FDG animation was created using an in-house modified version of the ForceAtlas2 class in fa2 Python package by saving all the intermediate states (published version only outputs the final state and discards all intermediates). The FDG coordinates at each iteration were plotted and the resulting graphs were assembled in a mp4 video format using VideoWriter in cv2 (v3.3.1) Python package.

Differential gene extraction and validation. Differential gene validation was done using a random forest classifier (RandomForestClassifier class in ensemble module of sklearn Python package v0.19.1, with 500 estimators, min_sample_split of 5, class weights set to the ‘balanced’ policy and all other parameters set to default). The random forest algorithm was chosen as it resembled the FACS gating hierarchy. Seventy percent of the data was used for training and 30% for test. Parameter tuning was performed on training data using grid search. To determine whether tissue-related transcriptome variations were present in equivalent immune populations between liver, skin and kidney, each equivalent population was taken in turn and grouped according to its tissue of origin. Seurat FindMarkers function was then applied in a pairwise manner between each tissue subset to produce a cell-type-specific list of genes marking each tissue subset. These were investigated in turn for biological relevance, with representative genes displayed using the VlnPlot function of Seurat.

DEGs from B cell pseudotime were studied for significant expression change across stage and differentiation state using a one-way ANOVA with Tukey’s multiple comparison test. DEGs displaying significant variance in ln-normalized expression were further studied for correlation to DEGs identified within all other cell-types across stage. B cell pseudotime DEGs with significantly correlated trends of expression to DEGs within other cell-types across stage (P < 0.05, two-tailed Pearson’s R at 95% confidence interval) were plotted in Prism (v8.1.2, GraphPad Software). All graphs presented in the manuscript were plotted using ggplot2 R package. Seurat implementation of ggplot2, matplotlib Python package, Prism (v8.1.2, GraphPad Software) or Flowjo (v10.14.1). Spot plots are shown throughout the manuscript, displaying scaled expression of ln-normalized counts.

Primary immunodeficiency gene list curation. Disease and genetic deficiency information was extracted from a previous study and manually annotated to include a representative symbol name for each disease-associated genetic defect for subsequent correlation with the liver dataset. Diseases implicated in primary immunodeficiency (PIDs) were divided according to the International Union of Immunological Societies (IUIS) major categories and screened across the liver scRNA-seq dataset. Three-hundred-and-fifteen unique genes were identified in the dataset from the 354 inborn errors of immunity highlighted in the article. For each disease category, a dot plot was generated using Seurat DotPlot function and ordered by highest expression across each gene and across each cell type, highlighting those cell types in each disease category that express the highest number of genes associated with a genetic defect.

CellPhoneDB analysis. CellPhoneDB v2.0 (www.cellphonedb.org) was used for the receptor–ligand analysis in Extended Data Fig. 4a. Significant (P < 0.05) receptor–ligand interactions between VCAM1 and EL macrophages and the two erythroid (early and mid) populations were displayed.

Whole-genome sequencing and fetal cell identification. To identify maternal cells present in our data, we combined the information from fetal whole-genome DNA sequencing with the single-cell RNA-seq data. For each sample, we measured the allele frequency in the fetal DNA of SNPs from the 1.000 genomes project falling within exons with a population allele frequency in excess of 1%. We then considered only those SNPs that are homozygous in the fetal DNA for follow up in the scRNA-seq data. A SNP was considered to be homozygous if its allele frequency in the fetal DNA was less than 0.2 or greater than 0.8 and had a false discovery rate-adjusted P value of less than 0.01 under a binomial test for the null hypothesis that the allele frequency in the DNA was in the range [0.3, 0.7].

The allele frequency of each of these SNPs with population allele frequency >1% that are known to be homozygous in the fetal DNA was then measured in each cell in the scRNA-seq data. Any deviations from homozygosity in the RNA-seq data must be a consequence of either sequencing errors, RNA editing or the genotype of the cell differing from the fetal DNA. For each cell, we calculated the total fraction of reads (selectable SNPs) differing from the expected genome-wide rate of deviations due to sequencing errors and RNA editing is less than or equal to 2%. For maternal cells, the expected genome-wide rate of deviation at these SNPs is equal to half the mean of the population allele frequency at the interrogated SNPs. Finally, for each cell we calculated the posterior probability of the cell being fetal or maternal assuming a binomial distribution with rate 2% for a fetal cell and half the mean of the population allele frequency for the maternal cell and assigned a cell as maternal or fetal if either posterior probability exceeded 99%; ambiguous otherwise. We validated this method using samples for which both the fetal and maternal DNA were available.

Imaging mass cytometry. Antibodies were conjugated to metals using the Fluidigm MaxPar conjugation kits and the associated method with the following modifications: the lanthanides were used at 1.5 mM and washed for a shorter duration (4 × 5 min) in W-buffer before elution. Ultrapure MilliQ water was used throughout for any dilutions and washes. Four-micrometre-thick formalin-fixed paraffin-embedded sections obtained from 8 and 15 PCW fetal liver tissue blocks were incubated at 60 °C for 1 h then dewaxed in xylene (Fisher). After rehydration through graded alcohols (Fisher) and a 5-min wash in water, the sections were sub - jected to a 15-min wash with citrate buffer (pH 6.0). Sections were then washed in water and PBS ( Gibco) and blocked with 3% BSA ( Sigma Aldrich) for 45 min. A mixture of eight metal-conjugated antibodies diluted in 0.5% BSA (see Supplementary Table 21 for antibody details), was added to the sections for overnight incubation at 4 °C in a humidified chamber. Slides were washed twice in 0.2% Triton X-100 diluted in PBS for 8 min and then twice in PBS for 8 min. To counterstain nucleated cells, sections were incubated with 312.5 nm (193 Ir) Intercalator-Ir ( Fluidigm) for 30 min at room temperature. Slides were then washed in water for 5 min, and allowed to air dry at room temperature before imaging on the Hyperion imaging mass cytometer. Using expected target cell frequencies from previous fluorescence flow cytometry data, region-of-interest (ROI) size was set to 2.8 mm × 3.8 mm. The ablation energy was set at 2 dB with a laser frequency of 200 Hz. Each session of ablation generated a .mcd image file containing information for every panorama and ROI measured whereby each 1-μm piece of tissue liberated by the laser was analyzed for ionic content on a per channel basis by time of flight. Single-cell segmentation and feature extraction was performed using CellProfiler (v3.1.5). Nuclei were identified using the IdentifyPrimaryObjects module, in which the input images were the sum of the DNA-stained iridium channels (191 and 193) constructed by the ImageAfterMath module. The diameter range set for nuclei identification was 4–15 pixel units. The ExpandOrShrink module was used to grow the nuclear segmentation area by three pixels to define the cellular area and the MeasureObjectIntensity module was used to determine the mean intensity for each cell object identified.

Light-sheet and confocal fluorescence microscopy. Male embryos at 5, 7 and 11 PCW that were deemed to be devoid of morphological anomalies were dissected after overnight fixation in 4% PFA. Whole-mount and cryosection immunostaining were performed as described previously, with the following conditions: tissue was washed with primary antibody buffer (see Supplementary Table 22 for antibody details) for 9 days at 37 °C with secondary antibody for 16 h at 37 °C using dedicated host species antibodies and reagent combination. TO-PRO-3 647 was used at 1:100 in whole embryos and 1:5,000 on cryosections. Whole-mount specimens
were solvent-cleared as described\textsuperscript{14}, and imaged in dibenzylether with a Milleniyi Lavigation Biotech ultramicroscope (Olympus MVX10 stereomicroscope and PCO Edge SCMOS CCD camera using the dedicated Inspector pro acquisition software. Four lasers (at 488, 561, 647 and 790 nm wavelengths) were used to generate light sheets. Imaris (v9.2, BitPlane) was used for image conversion and processing. To assess co-labelling at a cellular level the DBE-cleared specimens were further imaged using an upright confocal microscope Olympus FV-1000 with the following wavelengths: 488 nm (diode), 1,440,075 nm (diode), acquired using Olympus Fluaview 4.2 b and displayed in Imaris 9.2. Deconvolution was performed using NIH-FIJI. Photoshop (Adobe) was used to create panels. All raw files are available on demand through our dedicated online platform (www.transparent-human-embryo.com).

**Statistics and reproducibility.** For all analyses of fetal liver 3′ 10x data, n = 14 biologically independent samples were included. This includes Figs. 1a, 2a, 3a, 4b, 5a, 5b, 6a, 6b, Extended Data Figs. 1c, 2a, 3a–e, 4a, 5c, g–i, 6a–c, 6b, 7, 8. For all analyses of fetal liver 3′ 10x data by developmental stage. n = 4 – 7 PCW, n = 4 – 11 PCW, n = 3 – 12 – 14 PCW and n = 3 – 15 – 17 PCW biologically independent samples were used. This includes Figs. 1c, 4a, Extended Data Figs. 1d, e, 5a–c, f. For all analyses including 10x sequencing data of cord blood and adult bone marrow, n = 8 biologically independent samples of each were used. These analyses are shown in Fig. 6g and Extended Data Fig. 7b. For all scRNA-seq data shown, all barcodes of a given label from indicated tissues are shown, and no downsampling or subsetting was performed. The following cell numbers generated using scRNA-seq are displayed in each of the listed figures: Figs. 1a, b, c–e, h, 5g–i, 6c–e. All comparisons shown in Extended Data Fig. 7 was done by applying two-tailed Fisher’s exact tests to colony counts. Statistical analysis of differential gene expression was done using one-tailed Wilcoxon rank sum test with Bonferroni correction, including those shown in heatmaps and violin plots. In Fig. 6b, expression of genes in HSC/MPP 1 compared to each other cell cluster, and MEMP/neutrophil-myeloid progenitor/pre-pro-B cell compared to each other cell cluster was statistically significant unless specified as not significant (NS). All P < 0.001, except expression of KLF1 between HSC/MPP 1 vs HSC/MPP 2 (P = 0.00934), ILIR1 between HSC/MPP 1 vs HSC/MPP 2 (P = 0.00148), GATA2 between MEMP vs HSC/MPP 2 (P = 0.00802), BCL11A between HSC/MPP 1 vs HSC/MPP 2 (P = 0.00114), IL7 between HSC/ MPP 1 vs HSC/MPP 2 (P = 0.00124), BCL11A between HSC/MPP 1 vs HSC/MPP 2 (P = 0.00494) and CSFIR between neutrophil-myeloid progenitors vs HSC/ MPP 8 (P = 0.213). In Fig. 6g, P** < 0.001 when comparing expression of ANX1A, DUSP1, HLA-B and HSPO1 between samples. Expression of genes in fetal liver early lymphoid/T lymphocytes was compared across developmental stages and displayed in Extended Data Fig. 5b, c. No significant difference across stage was observed in the genes displayed in Extended Data Fig. 5b. The following comparisons shown in Extended Data Fig. 5c were significant: CD2 expression at 7–8 PCW vs 9–17 PCW (P < 0.0001) and 12–17 PCW (P < 0.001); TRDC expression at 7–8 PCW vs 9–17 PCW (P < 0.0001) and 11–17 PCW vs 12–17 PCW (P < 0.001); CD8A expression at 7–8 PCW vs 9–17 PCW (P = 0.00714); CD27 expression at 7–8 PCW vs 9–17 PCW (P < 0.001) and 9–11 PCW vs 12–17 PCW (P < 0.001); IL7R expression at 7–8 PCW vs 9–17 PCW (P < 0.0001); CD3D expression at 7–8 PCW vs 9–17 PCW (P < 0.0001); TRAC expression at 7–8 PCW vs 9–17 PCW (P < 0.0001) and 9–11 PCW vs 12–17 PCW (P < 0.0001); CD3D expression at 7–8 PCW vs 9–17 PCW (P < 0.0001); KLRB1 expression at 9–11 PCW vs 12–17 PCW (P < 0.001); TRAC expression at 7–8 PCW vs 9–17 PCW (P < 0.0001) and 9–11 PCW vs 12–17 PCW (P < 0.0001); PRF1 expression at 9–11 PCW vs 12–17 PCW (P < 0.0001); CD27 expression at 7–8 PCW vs 9–17 PCW (P = 0.00168) and 12–14 PCW vs 15–17 PCW (P = 0.00216); ICHEX expression at 7–8 PCW vs 9–17 PCW (P < 0.0001); CD3D expression at 7–8 PCW vs 9–17 PCW (P < 0.0001); S100B expression at 9–11 PCW vs 12–17 PCW (P < 0.0001). The following comparisons were statistically significant (P < 0.001) between macrophages from different tissues: CD14 expression in kidney vs liver and vs skin; CD68 expression in liver vs skin and vs kidney; CD163, RNASE1 and FISS1A expression between all tissues; CD14 expression in liver vs skin vs kidney and vs placenta, as shown in Extended Data Fig. 5a, is compared between tissues and no significant difference was observed. Extended Data Fig. 6a shows comparisons between fetal liver, skin and kidney for macrophages, monocytes, pDCs, DC1 and DC2. The following comparisons were statistically significant (P < 0.001) between macrophages from different tissues: CD14 expression in kidney vs liver and vs skin; CD68 expression in liver vs skin and vs kidney; S100A9 expression in liver vs kidney (P < 0.0001), and skin vs kidney (P = 0.0245); FCGR3A. expression in liver vs skin (P = 0.0004); POSTN expression in liver vs skin (P < 0.0001) and vs kidney (P < 0.0001), and skin vs kidney (P = 0.0411). No significant difference was observed when comparing genes in pDCs across tissues. CLEC9A expression was statistically significantly different in liver vs skin DC1 (P < 0.0001). The following comparisons were statistically significant between DC2 from different tissues: CD1C expression in skin vs liver (P < 0.0001) and virus kidney (P < 0.0001); CLEC10A expression in kidney vs liver (P < 0.0001) and vs skin (P < 0.0001); S100B expression in skin vs liver (P < 0.0001) and vs kidney (P = 0.0162); and FCER1A and CD83 expression between each tissue (P < 0.0001).

Statistical analysis of HSC colony assays shown in Fig. 6c–e and Extended Data Fig. 7 was done by applying two-tailed Fisher’s exact tests to colony counts. Figure 6c, d and Extended Data Fig. 7 show 125, 217 and 124 colonies from 7–8 PCW fetal liver samples, respectively. The number of colonies in each sample was n = 2 biologically independent samples per developmental stage. The number of colonies per sample is 93, 123, 93, 93, 79 and 40, which are shown in Extended Data Fig. 7c. The number of colonies between the following stages in Fig. 6c were statistically
significant: erythroid colonies in 7–8 PCW vs 15–17 PCW (P = 0.0238), erythroid/megakaryocyte/myeloid colonies in 7–8 PCW vs 15–17 PCW (P = 0.0294), NK colonies in 7–8 PCW vs. 15–17 PCW (P = 0.0357), and erythroid/myeloid colonies in 7–8 PCW vs 12–14 PCW (P = 0.0188) and 15–17 PCW (P < 0.001), and 12–14 PCW vs 15–17 PCW (P = 0.0252). The number of erythroid-containing colonies was significant between each stage shown in Fig. 6d (P < 0.001). The number of colonies that differentiated along three lineages was significant between 7–8 PCW vs 12–14 PCW (P = 0.0041), and 7–8 PCW vs 15–17 PCW (P = 0.0027). Figure 6e shows 141, 74 and 124 colonies from 7–8 PCW, 12–14 PCW and 15–17 PCW fetal liver samples, respectively, from n = 2 biologically independent samples per development stage. The number of B cell-forming colonies in Fig. 6e was significant between 7–8 PCW vs 12–14 PCW (P = 0.0014) and 15–17 PCW (P = 0.0044). Extended Data Fig. 7d, e shows 163, 196 and 182 colonies from n = 3 7–8 PCW, n = 2 12–14 PCW and n = 2 15–17 PCW biologically independent fetal liver samples, respectively, on the left, and 42, 74, 49, 99, 59 and 123 colonies by individual sample on the right. The number of erythroid colonies compared to all other types shown in Extended Data Fig. 7d was statistically significant between 12–14 PCW and 15–17 PCW (P = 0.0307). The number of erythroid-containing colonies was significant between 7–8 PCW vs 15–17 PCW (P = 0.0013), and 12–14 PCW vs 15–17 PCW (P = 0.0497), as shown in Extended Data Fig. 7e. Extended Data Fig. 7i shows 125, 217 and 124 colonies from 7–8 PCW, 12–14 PCW and 15–17 PCW fetal liver samples, respectively, from n = 2 biologically independent samples per development stage. Extended Data Fig. 7g shows 141, 74 and 124 colonies from 7–8 PCW, 12–14 PCW and 15–17 PCW fetal liver samples, respectively, from n = 2 biologically independent samples per development stage. The percentage of NK-containing colonies was statistically significant in 7–8 PCW vs 15–17PCW (P = 0.0032), and 12–14 PCW vs 15–17 PCW (P = 0.0115). Flow cytometric analysis of cell cycle phases, as shown in Fig. 6g and Extended Data Fig. 7i was performed on cells from n = 3 7–8 PCW and n = 3 12–16 PCW biologically independent fetal liver samples, and n = 2 biologically independent cord blood samples. One-way ANOVA with Tukey’s multiple comparison test was used to determine statistical significance between (7–8 PCW and 12–14 PCW) and samples (fetal liver and cord blood). The percentage of CD34+CD38− cells in GC was significantly higher in 12-14 PCW livers compared with 7–8 PCW livers (P = 0.0136).

Cell cycle phases determined by transcriptome analysis of fetal liver cells, fetal skin cells, and fetal kidney cells are shown in Extended Data Fig. 3h. Statistical significance of the proportion of MEMPs and megakaryocytes in each cell cycle phase was compared between fetal liver, skin and kidney using a Kruskal–Wallis test with Dunn’s post hoc test. Statistical significance of the proportion of mid and late erythroblasts, and mast cells in each cell cycle phase was compared between fetal liver and skin using two-tailed Mann–Whitney tests. The following comparisons were statistically significant: megakaryocytes in fetal liver vs fetal kidney in G1 (P = 0.0317), G2M (P = 0.0317) and S (P = 0.0319); megakaryocytes in fetal liver vs fetal skin in S (P = 0.0039); mid erythroblasts in fetal liver vs skin in G1 (P = 0.0031) and G2M (P < 0.0001); late erythroblasts in fetal liver vs fetal skin in G1 (P = 0.0021) and S (P < 0.0001); and mast cells in fetal liver vs fetal skin in G1 (P = 0.0248) and S (P = 0.0337).

Statistical comparison of the proportion of MEMPs, mid and late erythroblasts, megakaryocytes and mast cells expressing MKI67 in fetal liver vs NLT (skin and kidney) was performed using two-tailed Mann–Whitney tests. This is displayed in Extended Data Fig. 3h, where the following comparisons were statistically significant: megakaryocytes in fetal liver vs fetal NLT (P = 0.007), mid erythroblasts in fetal liver vs fetal skin (P = 0.0305), and late erythroblasts in fetal liver vs fetal skin (P = 0.0368).

Statistical comparison of the proportion of fetal liver HSC/MPP expressing MKI67 compared to the percent of fetal liver MEMP, pre pro-B cells, neutrophil-myoeloid progenitors, monocyte precursors, pDC precursors and DC precursors, yolk sac progenitors, and HSC from cord blood and adult bone marrow expressing MKI67 was performed using a Kruskal–Wallis test with Dunn’s post hoc test. This is displayed in Extended Data Fig. 7i, in which the percentage of MKI67 expressing fetal liver HSC/MPPs was statistically significant when compared with the following populations: fetal liver MEMPs (P = 0.0180), monocyte precursors (P < 0.0001), DC precursors (P < 0.0001), cord blood HSC (P < 0.0001) and adult bone marrow HSC (P = 0.0076).

Extended Data Fig. 1g displays a flow cytometric analysis of the frequency of B cells in the CD34− cells from n = 13 6–9 PCW, n = 13 9–12 PCW, n = 14 12–15 PCW and n = 9 15–19 PCW biologically independent fetal liver samples. Statistical significance across the developmental stages was compared using a Kruskal–Wallis test with Dunn’s post hoc test. The following comparisons were statistically significant: 6–9 PCW vs 12–15 PCW (P < 0.0001), 6–9 PCW vs 15–19 PCW (P = 0.0003), 9–12 PCW vs 12–15 PCW (P = 0.0157) and 9–12 PCW vs 15–19 PCW (P = 0.0287).
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**Competing interests** A. Regev is a founder and equity holder of Celsius Therapeutics and an SAB member of Neogene Therapeutics, ThermoFisher Scientific and Syros Pharmaceuticals.

**Additional information**

**Supplementary information** is available for this paper at https://doi.org/10.1038/s41586-019-1652-y.

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Extended Data Fig. 1 | Single-cell transcriptome map of fetal liver.

**a.** Fetal skin and kidney haematopoietic cells visualized by UMAP. Colours indicate cell state. Inset, colours indicate tissue type. **b.** UMAP visualization of yolk sac haematopoietic cells. Colours indicate cell state. Inset, colours indicate location within yolk sac. **c.** UMAP visualization of 3′ liver 10x cells after batch correction, coloured by sample. **d.** UMAP visualization (top) of 3′ 10x liver sample sex mixing grouped by developmental stage, and violin plots (bottom) showing ln-normalized median expression of XIST (green) and RPS4Y1 (purple), which mark female and male samples, respectively. **e.** UMAP visualization of fetal liver composition by developmental stage. Colours indicate cell state. **f.** UMAP visualization of fetal liver cells profiled using Smart-seq2. Colours indicate cell states as shown in e, g. Frequency (mean ± s.e.m.) of B cells in the CD34− cells detected in 6–19 PCW fetal livers by flow cytometry (*P < 0.05, ***P = 0.003 and ****P < 0.001).
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | Transcriptome validation of fetal liver cells.

a, Assessment of 48 genes from the 4,471 highly variable genes by using a random forest classifier to assign cell labels, where ‘true cell label’ indicates the manual annotation based on the full list of variable genes. b, Comparison of representative mini bulk RNA-seq data (in coloured triangles) and liver EI populations (early, mid and late erythroids, VCAM1+ EI macrophages), Kupffer cells and endothelium validated by Smart-seq2 (SS2) (in colour) overlaid on whole liver SS2 populations (grey). c, Dot plot showing representative median-scaled ln-normalized gene expression of 100 FACS-isolated liver cells based on marker gene expression in Fig. 2a. Gene expression indicated by spot size and colour intensity. d, Dot plot showing median-scaled ln-normalized gene expression of FACS-sorted single cells from liver EI populations (early, mid and late erythroids, VCAM1+ EI macrophages), Kupffer cells and endothelium shown as coloured dots in b based on marker gene expression in Fig. 2a. Gene-expression frequency (per cent of cells within cell type expressing the gene) indicated by spot size and expression level by colour intensity. e, Overlay pseudo-colour Hyperion representative images for 8 PCW and 15 PCW fetal liver. Far left images are shown at 5× magnification with zoom of insets on right at 20× magnification (1 μm per pixel). Asterisks indicate bile ducts.
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3 | Fetal liver and NLT haematopoiesis. a, PAGA analysis of fetal liver HSC/MPP, erythroid, megakaryocyte and mast cell lineages from Fig. 3a. Lines show connections; line thickness corresponds to the level of connectivity (low (thin) to high (thick) PAGA connectivity). b, Heat map showing min — max normalized expression of statistically significant ($P < 0.001$), dynamically variable genes from pseudotime analysis for erythroid, megakaryocyte and mast cell inferred trajectories. Transcription factors in bold, asterisks mark genes not previously implicated for the respective lineages. c, FDG visualization of fetal liver, skin and kidney HSC/MPP, MEMP, erythroid, megakaryocyte and mast cell lineages. d, Heat map showing the scaled ln-expression of selected marker genes in fetal liver, NLT and yolk sac subsets. e, PAGA connectivity scores of HSC/MPP, erythroid, megakaryocyte and mast cell lineages between fetal liver, skin, kidney (K) and yolk sac. f, Representative immunohistochemical staining of sequential sections of 8 PCW fetal skin for endothelium (CD34$^+$) and erythroblasts (nucleated and GYPA$^+$), nuclei stained with blue alkaline phosphatase. Zoom in of insets (right) bordered with black (top) indicate nucleated cells stained positive for GYPA within CD34$^+$ blood vessels, and those bordered with red (bottom) indicate nucleated GYPA$^+$ cells outside CD34$^+$ blood vessels. Scale bars, 100 μm. g, Representative confocal fluorescence microscopy of embryo (5 PCW) hand skin. Scale bar, 10 μm; red, TO-PRO-3 nuclei; green, GYPA (see also Supplementary Video 2 showing light-sheet fluorescence microscopy). The arrowhead indicates extravascular nucleated erythroid cells. h, Stacked bar plots (left) showing percentage (mean ± s.d.) of fetal liver (red), skin (blue) and kidney (green) HSC/MPP, MEMP, erythroid, megakaryocyte and mast cells in each stage of the cell cycle (G1 (navy), G2M (blue) and S (white) phase), and ln-normalized median expression of MKI67 transcript (right) in corresponding liver vs NLT cell types (total percent of MKI67-expressing cells shown above plots; each dot represents a single cell). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.005$. 
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Investigation of interactions between fetal liver macrophages and erythroid cells. 

a, Representative immunohistochemical staining of fetal liver for erythroblasts and macrophages with GYPA and CD68, respectively. Scale bar, 50 μm. Statistically significantly ($P < 0.05$) enriched receptor–ligand interactions from CellPhoneDB between VCAM1+ EI macrophages (purple) and two erythroid populations (early and mid; red) ($n = 14$ biologically independent samples). Asterisks indicate protein complexes. Violin plots show ln-normalized median gene-expression value of VCAM1 and ITGA4 in cells analysed by CellPhoneDB (indicated by # in dot plot).

b, Representative immunohistochemical staining of sequential sections of 8 PCW fetal liver for VCAM1+ EI macrophages (VCAM1+) and CD49d+ GYPA+ cells with nuclei stained using blue alkaline phosphatase. Right, zoom in of insets, with coloured arrows indicating erythroblast (yellow) and VCAM1+ EI macrophage (purple). Scale bars, 100 μm.

c, Representative gating strategy used to visualize fetal liver erythroid cells, VCAM1+ EI macrophages, Kupffer cells, monocyte-macrophages and mast cells.

d, Bright field (BF), VCAM1 (CD106), CD34, CD45, KIT (CD117), GYPA, CD14 and HLA-DR images for each cell type within gates shown in c. e, Representative bright-field images of cells found within the single-cell and doublet gates.

f, Bar plots showing the proportion of each cell type within the single-cell gate (white) or doublet gate (grey) (mean ± s.d.); *$P = 0.0194$. g, Comparison of macrophage and erythroid gene expression in mouse macrophages (red) and EI macrophages (blue), $n = 3$ from ref. 20.
Extended Data Fig 5 | See next page for caption.
Extended Data Fig 5 | Lymphoid lineages in fetal liver and NLT.

a, PAGA analysis of fetal liver HSC/MPP and lymphoid cell types from Fig. 1b showing changes over four developmental stages. Lines symbolize connection; line thickness corresponds to the level of connectivity (low (thin) to high (thick) PAGA connectivity).

b, c, Feature plots (b) and violin plots (c) showing ln-normalized median expression of selected known NK, ILC and T cell genes over gestation for early lymphoid/T lymphocyte cluster; **P < 0.001, ***P < 0.005 and ****P < 0.0001.

d, Dot plot showing median-scaled ln-normalized median expression of V(D)J transcripts in fetal liver lymphoid cell types. Gene expression indicated by spot size and colour intensity.

e, Heat map showing normalized expression of statistically significant, dynamically variable genes from pseudotime analysis for B cell lineage inferred trajectory (likelihood ratio test). Transcription factors are in bold. Morphology of liver pro-B and pre-B cells and B cells by Giemsa stain after cytopsin.

f, In-normalized expression (mean ± s.e.m.) of TNFSF13B in Kupffer cells and NFKBIA in HSC/MPPs and cells in the B cell lineage across four developmental stages spanning 6–17 PCW; trend lines showing linear regression.

g, PAGA connectivity scores of HSC/MPP and lymphoid cells from fetal liver, skin, kidney and yolk sac.

h, ln-normalized median expression of selected known NK (top) and ILC precursor (bottom) marker genes and selected DEGs between liver (red), skin (blue) and kidney (green) visualized by violin plots (***P < 0.005 and ****P < 0.001).

i, Violin plots showing ln-normalized median expression of selected known ILC and NK cell genes expressed in ILC precursors from fetal liver, skin and kidney.
Extended Data Fig. 6  | Tissue signatures in developing myeloid cells.  

a, Diffusion map of fetal liver HSC/MPP, progenitors and precursors from Fig. 1b.  
b, Heat map showing min — max normalized expression ($P < 0.001$) of dynamically variable genes from pseudotime analysis for monocyte, DC1 and DC2 inferred trajectories. Transcription factors in bold, asterisks mark genes not previously implicated for the respective lineages.  
c, Heat map visualization comparing scaled expression of the top marker genes of decidua-placenta (red), fetal liver (black) and yolk sac (purple) progenitor and myeloid populations.  
d, PAGA connectivity scores of HSC/MPP and myeloid cells from fetal liver, skin and kidney.  
e, ln-normalized median expression of three known marker genes and three DEGs in corresponding myeloid populations across fetal liver, skin and kidney visualized by violin plots ($^*P < 0.05$, $^{***}P < 0.005$ and $^{****}P < 0.0001$).
Extended Data Fig. 7 | See next page for caption.
Extended Data Fig. 7 | HSC/MPP differentiation potential by gestation. 
a, Experimental design for single-cell transcriptome and culture of fetal liver cells from the representative FACS gates illustrated. 
b, Alignment of 349 scRNA-seq-profiled cells from FACS gates in a with 10x-profiled HSC/MPPs and early progenitors visualized using FDG; point shape corresponds to sequencing type (triangle, SS2 plate data; circle, 10x data). 
c, Stacked bar plot of all different types of colonies generated by single HSC pool gate cells (gate defined in a). 
d, Stacked bar plot of all different types of colonies generated by single HSC pool gate cells without MS5 stroma layer (gate defined in a) by stage (left) and in individual samples (right), *P < 0.05. 
e, Percentage of colonies generated by single HSC pool cells without MS5 stroma layer containing erythroid cells (sum of ery, ery–meg, ery–meg–my and ery–my colonies shown in c); **P < 0.01. 
f, Percentage of colonies from single-cell culture (shown in Fig. 6c) that differentiated along three lineage (defined as sum of ery, NK and my, and ery, meg and my colonies) branches (****P < 0.005). 
g, Percentage of colonies containing NK cells following B/NK optimized culture of ten cells from the HSC pool gate (*P < 0.05 and **P < 0.01). 
h, Percentage (mean ± s.e.m.) of HSC/MPPs and early progenitors in fetal liver, yolk sac, cord blood and adult bone marrow expressing MKI67 (*P < 0.05, **P < 0.01 and ****P < 0.001). 
i, Percentage (mean ± s.e.m.) of CD34+CD38– and CD34+CD38+ cells in the indicated cell cycle phases (right) as determined by flow cytometry analysis (left; representative plot of n = 8 biologically independent samples) (G0, Ki67–DAPI–; G1, Ki67–DAPI+; S–G2–M, Ki67–DAPI+ (left)).
Extended Data Fig. 8 | Expression of known PID-linked genes in fetal liver. Dot plots showing relative expression of genes known to be associated with major PID disease categories in fetal liver cell types from Fig. 1b. Gene-expression frequency (per cent of cells within the cell type expressing the gene) is indicated by spot size and expression level is indicated by colour intensity.
Extended Data Fig. 9 | FACS gating strategy for scRNA-seq analysis.

a, Gating strategy used to isolate cells for droplet (10x) and plate-based scRNA-seq (Smart-seq2) for samples F2–F17. b, Gating strategy used to isolate cells for cytospins, scRNA-seq (Smart-seq2) and 100-cell RNA-seq. c, Flow cytometry gating strategy used to identify the colonies cultured in vitro from single cells as shown in Fig. 6c. d, Flow cytometry gating strategy used to identify B and NK colonies cultured in vitro from 10 cells as shown in Fig. 6c.
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

No software was used for data collection.

Data analysis

Alignment, quantification and quality control of scRNA-seq data

Droplet-based (10x) sequencing data was aligned and quantified using the Cell Ranger Single-Cell Software Suite (version 2.0.2, 10x Genomics Inc) using the GRCh38 human reference genome (official Cell Ranger reference, version 1.2.0). Smart-seq2 sequencing data was aligned with STAR (version 2.5.1b), using the STAR index and annotation from the same reference as the 10x data. Gene-specific read counts were calculated using htseq-count (version 0.10.0). Cells with fewer than 200 detected genes and for which the total mitochondrial gene expression exceeded 20% were removed. Genes that were expressed in fewer than 3 cells were also removed. We detected on average ~3,000 genes per cell with the 10x Genomics platform and ~6,000 genes with the Smart-seq2 protocol.

Doublet detection

Doublets were detected with an approach adapted from Pijuan-Sala et al. In the first step of the process, each 10x lane was processed independently. Scrublet was run, obtaining per-cell doublet scores. The standard Seurat-inspired Scapny processing pipeline was performed up to the clustering stage, using default parameters. Each cluster was subsequently separately clustered again, yielding an over clustered manifold, and each of the resulting clusters had its Scrublet scores replaced by the median of the observed values. The resulting scores were assessed for statistical significance, with p-values computed using a right-tailed test from a normal distribution centred on the score median and a MAD-derived standard deviation estimate. The MAD was computed from above-median values to circumvent zero-truncation. The p-values were FDR-corrected with the Benjamini-Hochberg procedure, and a significance threshold of 0.1 was imposed. In the second step of the process, all 10x lanes for a single tissue were pooled together and the Seurat-inspired Scapny processing was repeated, with the addition of Harmony7 with a theta of 3 for batch correction between the lanes before the neighbour graph identification step. The joint manifold was clustered, and the frequency of identified doublets was computed. The same statistical framework as in the first step was used to identify clusters significantly enriched in doublets, which were subsequently flagged as doublets in their entirety and removed.

Clustering and annotation
Downstream analysis included data normalisation (NormalizeData, LogNormalize method, scaling factor 10000), data feature scaling (ScaleData), variable gene detection (FindVariableGenes), PCA (RunPCA, from variable genes) and Louvain graph-based clustering (FindClusters, data dimensionality reduction using PCA, clustering parameter res (30)) performed using the R package Seurat (version 2.3.4). Cluster cell identity was assigned by manual annotation using known marker genes and computed differentially expressed genes (DEGs) using FindAllMarkers function in Seurat package (Wilcoxon rank sum test, p-values adjusted for multiple testing using the Bonferroni correction; Supplementary Table 3). For computing DEGs all genes were probed provided they were expressed in at least 25% of cells either of the two populations compared and the expression difference on a natural log scale was at least 0.25. Manual annotation was performed iteratively, which included validating proposed cell labels with known markers and further investigating clusters whose gene signatures indicated additional diversity.

Clustering and cell type assignment for fetal liver data was assessed using two additional clustering methods (not shown): Agglomerative clustering (with Ward linkage and Euclidean affinity) and Gaussian mixture (AgglomerativeClustering class from cluster module and GaussianMixture from mixture module in sklearn version 0.19.1 Python 3.6.3). Consensus agreement between the 3 clustering methods was measured by Rand index and adjusted mutual information implemented in the metrics module in sklearn package. The Rand Index scores were 0.89 and 0.85 for Agglomerative and Gaussian Mixture clustering methods respectively.

After annotation was completed, a cell type classifier was built by training an SVM on labelled fetal liver scRNA-seq data with grid search for parameter optimization based on training data. 70% of the data was used for training and the other 30% for test. The SVM was previously compared in terms of accuracy and recall with a random forest and logistic regression classifiers trained on the same data. Out of the 3 classifiers the SVM was chosen due to showing a mean accuracy and weighted mean recall of 95%. Random forest showed 89% for both precision and recall (Supplementary Table 7). The SVM classifier was used for automatic annotation of the Smart-seq2 and mini bulk RNA sequencing data sets to allow identification of biologically meaningful clusters and DEG computation.

Data generated from fetal skin, kidney and yolk sac was pre-processed, normalised, clustered and manually annotated, in parallel with, and using the same pipeline as, the liver data. Skin and kidney data were combined using the MergeSeurat function. Clusters characterised by differentially expressed immune gene markers were extracted from the NLT dataset for subsequent comparative analysis with liver-derived immune populations. Human cord blood and adult bone marrow datasets were downloaded from Human Cell Atlas data portal (https://preview.data.humancellatlas.org/). These were processed using the same approach as described above, followed by manual annotation. Decidua and placental data from Vento-Tormo et al., were downloaded from ArrayExpress record E-MTAB-6701 (https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-6701/).

Data integration We used Harmony data integration to correct for batch effect between sample identities. The average kBET rejection rate statistically significantly improved from 0.735 to 0.471 (Supplementary Table 8) following Harmony data integration (p-value 3.8e-3 in Kolmogorov–Smirnov test and p-value 8.8e-6 in Wilcoxon signed-rank test). The manifold was subjected to re-clustering using Harmony adjusted PCs with parameters as mentioned above in “Clustering and annotation”. Cell type classifications were then ascertained through re-annotation of the clusters derived from Harmony adjusted PCs to produce the final annotation.

Changes in cell proportions over development Comparison of cell proportions across gestational stages was assessed using negative binomial regression on cell number data using the glm.nb function in the R MASS package. Cell numbers were corrected for CD45-/CD45+ FACS sorted ratio (Supplementary Table 1) prior to applying negative binomial regression.

Dimensionality reduction and trajectory analysis Dimensionality reduction methods included tSNE (Seurat, computed from the first 20 PCs, Barnes-Hut fast computation), UMAP (Python UMAP package, 5 nearest neighbours, correlation metric, minimum distance 0.3, computed from the first 20 PCs), FDG (ForceAtlas2 class from fa2 Python Package, Barnes-Hut implementation for faster computation with theta 0.8, 2000 iterations) and partition-based approximate graph abstraction (PAGA) (paga in scanny Python package version 1.2.2). Development trajectories were inferred by comparing FDG, PAGA and diffusion map plots. Inferred trajectory analysis included computing diffusion map (scanny tdiffmap with 20 components), pseudotime (scanny t.dpt setting the earliest known cell type as root) and variable genes across pseudotime. Order of cells in pseudotime was statistically significant using Kruskal-Wallis test (p < 1x10-7).

Comparisons of trajectories across stage were performed by subsetting liver dataset by stage using SubsetData function, computing dimension reduction coordinates, batch correcting by sample using Harmony, and plotting PAGA and FDG by stage. Cell type comparisons across tissue involved subsetting for cell types of interest using SubsetData function, merging cross-tissue datasets using MergeSeurat function, and processing data using the same approach as for the liver and NLT datasets. Harmony batch correction was then performed by tissue type, with results presented as combined UMAPs, FDGs and PAGA score heatmaps.

Dynamically expressed genes across pseudotime Genes that vary across pseudotime were calculated using DifferentialGeneTest function in Monocle in R (version 2.6.4) and a cut-off of adjusted p-value < 0.001 applied. This was applied on the entire pseudotime range and also on the pseudotime intervals specific to each cell type in order to avoid limitation to the genes characterised by monotonic changes across the inferred trajectory. Expression of pseudotime variable genes were min-max normalized prior to visualization and annotated based on each gene’s involvement in relevant cell-specific functional modules or hallmark functional pathways from MSigDB v6.2, a curated molecular signature database66. Peak expression for each gene over pseudotime was calculated and grouped into ‘Early’, ‘Mid’ or ‘Late’ categories. For visualization purposes, the resulting gene lists were minimized by ordering them from those present in the most selected functional pathways to least, as well as ensuring coverage across pseudotime. These genes were manually compared against current literature to determine if they have known functional or cell type associations. The top 20-25 genes in each list were displayed using the ggplot2 package. Transcription factors were marked within the dataset based on AnimalTFDB transcription factor prediction database. The full pseudotime gene list is available in the interactive files accompanying diffusion maps.

Visualization by animated force-directed graph representation The FDG animation was created using an in-house modified version of the ForceAtlas2 class in fa2 Python package by saving all the intermediate states (published version only outputs the final state and discards all intermediates). The FDG coordinates at each iteration were plotted and the resulting graphs were assembled in a mp4 video format using VideoWriter in cv2 (version 3.3.1) Python package.
Differential gene validation was done using a random forest classifier (RandomForestClassifier class in ensemble module of sklearn Python package v0.19.1, with 500 estimators, min_sample_split of 5, class weights set to the “balanced” policy and all other parameters set to default). The Random Forest algorithm was chosen as it resembled the FACS gating hierarchy. 70% of the data was used for training and 30% for test. Parameter tuning was performed on training data using grid search. To determine whether tissue-related transcriptome variations were present in equivalent immune populations between liver, skin and kidney, each equivalent population was taken in turn and grouped according to its tissue of origin. Seurat FindMarkers function was then applied in a pair-wise manner between each tissue subset to produce a cell type-specific list of genes marking each tissue subset. These were investigated in turn for biological relevance, with representative genes displayed using VinyPlot function of Seurat.

DEGs from B-cell pseudotime were studied for significant expression change across stage and differentiation state using a one-way ANOVA with Tukey’s multiple comparison test. DEGs displaying significant variance in ln-normalised expression were further studied for correlation to DEGs identified within all other cell-types across stage. B-cell pseudotime DEGs with significantly correlated trends of expression to DEGs within other cell-types across stage (p-value <0.05, Two-tailed Pearson’s R at 95% CI) were plotted in Prism (v8.1.2, GraphPad Software). All graphs presented in the manuscript were plotted using ggplot2 R package, Seurat implementation of ggplot2, matplotlib Python package, Prism (v.8.1.2, GraphPad Software) or Flowjo (v10.4.1). Spot plots are shown throughout the manuscript, displaying scaled expression of In-normalized counts.

Primary immunodeficiency (PID) gene list curation
Disease and genetic deficiency information was extracted from Picard et al. and manually annotated to include HGNC symbol names for each disease-associated genetic defect for subsequent correlation with the liver dataset. Diseases implicated in PID were divided according to the International Union of Immunological Societies (IUIS) major categories and screened across the liver scRNA-seq dataset. 315 unique genes were identified in the dataset from the 354 inborn errors of immunity highlighted in the article. For each disease category a dot plot was generated using Seurat DotPlot function and ordered by highest expression across each gene and across each cell type, highlighting those cell types in each disease category which express the highest number of genes associated with a genetic defect.

CellPhoneDB analysis
CellPhoneDB v2.0 (www.cellphonedb.org) was used for the receptor-ligand analysis in Figure 3d. Significant (p < 0.05) receptor-ligand interactions between VCAM1+ Erythroblastic Island macrophages and the two erythroid (early and mid) populations were displayed.

Whole genome sequencing and fetal cell identification
To identify maternal cells present in our data we combined the information from fetal whole genome DNA sequencing with the single cell RNA-seq data. For each sample we measured the allele frequency in the fetal DNA of SNPs from the 1000 genomes project falling within exons with a population allele frequency in excess of 1%. We then consider only those SNPs which are homozygous in the fetal DNA for follow up in the scRNA-seq data. A SNP was considered to be homozygous if its allele frequency in the fetal DNA was less than 0.2 or greater than 0.8 and had an FDR adjusted p-value of less than 0.01 under a binomial test for the null hypothesis that the allele frequency in the DNA was in the range [0.3,0.7].

The allele frequency of each of these SNPs with population allele frequency > 1% that are known to be homozygous in the fetal DNA was then measured in each cell in the scRNA-seq data. Any deviations from homozygosity in the RNA-seq data must be a consequence of either sequencing errors, RNA editing, or the genotype of the cell differing from the fetal DNA. For each cell, we calculated the total fraction of reads at the SNPs (selected as described above) that differ from the fetal genotype. We then assume that the genome-wide rate of deviations due to sequencing errors and RNA editing is less than or equal to 2%. For maternal cells, the expected genome wide rate of deviation at these SNPs is equal to half the mean of the population allele frequency at the interrogated SNPs. Finally, for each cell we calculated the posterior probability of the cell being fetal or maternal assuming a binomial distribution with rate 2% for a fetal cell and half the mean of the population allele frequency for the maternal cell and assign a cell as: maternal/fetal if either posterior probability exceeds 99%, ambiguous otherwise. We validated this method using samples for which both the fetal and maternal DNA were available.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

**Data**

Policy information about availability of data
All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Raw sequencing data, expression count data with cell classifications are deposited at ArrayExpress: experiments: E-MTAB-7407.

**Field-specific reporting**

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
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For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf
Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size
Determined based on prior literature and Human Cell Atlas recommendations as per Human Cell Atlas white paper (www.humancellatlas.org/news/13)

Data exclusions
Any exclusions are stated in the quality control checks for single cell RNA-seq data in Methods.

Replication
Similar results were obtained using orthogonal methodological approaches including experiments performed across two laboratories.

Randomization
Samples were randomly allocated to this study through HDBR

Blinding
N/A

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

**Materials & experimental systems**

- n/a Involved in the study
  - [ ] Antibodies
  - [ ] Eukaryotic cell lines
  - [x] Palaeontology
  - [x] Animals and other organisms
  - [ ] Human research participants
  - [x] Clinical data

**Methods**

- n/a Involved in the study
  - [x] ChIP-seq
  - [x] Flow cytometry
  - [x] MRI-based neuroimaging

**Antibodies**

Antibodies used
All antibody details (clone and manufacturer) are included in Supplementary methods (Tables 8-14) and also listed below:

- Antibody Clone
- Manufacturer

- CD45 PerCP-Cy5.5 2D1 BD Bioscience
- HLA-DR APC L243 BD Bioscience
- CD34 PE 8G12 BD Bioscience
- CD45 APC-H7 2D1 BD Bioscience
- HLA-DR BV785 L243 Biolegend
- CD14 APC-Cy7 MøP9 BD Bioscience
- CD34 APC S81 BD Bioscience
- CD45 BUV395 H330 BD Bioscience
- CD56 PE NCAM16.2 BD Bioscience
- CD106 FITC 51-10C9 BD Bioscience
- GYPA BV605 GA-R2 BD Bioscience
- CD14 PE CF594 MøP9 BD Bioscience
- CD34 APC-Cy7 S81 Biolegend
- CD41 AF700 HIP8 Biolegend
- CD56 PE NCAM16.2 BD Bioscience
- CD94 APC REA113 Miltenyi
- CD117 PE-Cy7 104D2 Biolegend
- CD161 PerCP-Cy5.5 HP-3G10 Biolegend
- CD1c AF700 L161 Biolegend
- CD45RA BV510 H100 Biolegend
- CD20 FITC L27 BD Bioscience
- CD38 PerCP-Cy5.5 HB7 Biolegend
- CD117 PE-Cy7 104D2 Biolegend
- CD123 BUV395 7G3 BD Bioscience
- CLEC9A PE 8F9 Biolegend
- CD5 FITC SK7 BD Bioscience
- CD11c BV421 B-Ly6 BD Bioscience
- CD14 PE Dazzle HCD14 Biolegend
- CD16 FITC NKP15 BD Bioscience
- CD19 FITC 4G7 BD Bioscience
- CD38 PerCP-Cy5.5 HB-7 Biolegend
- CD49f PE-Cy7 GoH3 eBioscience
Validation

Imaging antibodies were validated against appropriate positive and negative tissue controls. Flow antibodies were validated by the manufacturer. Our flow cytometry and imaging mass cytometry data adhere to the information standards for MiFlowCyt for Flow/Mass cytometry (https://onlinelibrary.wiley.com/doi/pdf/10.1002/cyto.a.20623).

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)  MS5 - DSMZ, Germany

Authentication  None of the cell lines used were authenticated

Mycoplasma contamination  Cell lines were not tested for mycoplasma contamination

Commonly misidentified lines

(See ICLAC register)  Name any commonly misidentified cell lines used in the study and provide a rationale for their use.
Human research participants

Policy information about studies involving human research participants

Population characteristics
All embryo and fetal tissues were between 4 and 17 post conception weeks. For the liver samples we collected six male and nine female. All tissues were normal as determined by chromosomal assessment by QPCR to exclude the most commonly seen chromosomal abnormalities.

Recruitment
Human fetal tissues were obtained from the MRC/Wellcome Trust-funded Human Developmental Biology Resource (HDBR; http://www.hdbr.org) with appropriate written consent and approval from the Newcastle and North Tyneside NHS Health Authority Joint Ethics Committee (08/H0906/2145). HDBR is regulated by the UK Human Tissue Authority (HTA; www.hta.gov.uk) and operates in accordance with the relevant HTA Codes of Practice. Embryos and fetal specimens used for light sheet fluorescence microscopy were obtained with written informed consent from the parents (Gynecology Hospital Jeanne de Flandres, Lille, France) with approval of the local ethic committee (protocol NPFS16-002). Tissues were made available in accordance with the French bylaw (Good practice concerning the conservation, transformation and transportation of human tissue to be used therapeutically, published on December 29, 1998). Permission to utilize human tissues was obtained from the French agency for biomedical research (Agence de la Biomedecine #2016-841, Saint-Denis La Plaine, France).

Ethics oversight
Newcastle and North Tyneside NHS Health Authority Joint Ethics Committee

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation
All tissues were processed immediately after isolation using the same protocol. Tissue was transferred to a sterile 10mm2 tissue culture dish and cut into <1mm3 segments before being transferred to a 50mL conical tube. Tissue was digested with 1.6mg/mL collagenase type IV (Worthington) in RPMI (Sigma-Aldrich) supplemented with 10%(v/v) heat-inactivated fetal bovine serum (Gibco), 100U/mL penicillin (Sigma-Aldrich), 0.1mg/mL streptomycin (Sigma-Aldrich), and 2mM L-Glutamine (Sigma-Aldrich) for 30 minutes at 37°C with intermittent shaking. Digested tissue was passed through a 100μm filter, and cells collected by centrifugation (500g for 5 minutes at 4°C). Cells were treated with 1X RBC lysis buffer (eBioscience) for 5 minutes at room temperature and washed once with flow buffer (PBS containing 5%(v/v) FBS and 2mM EDTA) prior to counting. Antibody panels were designed to allow enrichment of cell fractions for sequencing and cell types validation. An antibody cocktail was prepared fresh by adding 3μL of each antibody in 50μL Brilliant Stain Buffer (BD) per tissue. Cells (≤10x106) were resuspended in 50-100μL flow buffer and an equal volume of antibody mix was added to cells from each tissue. Cells were stained for 30 minutes on ice, washed with flow buffer and resuspended at 10x106cells/mL. DAPI (Sigma-Aldrich) was added to a final concentration of 3μM immediately prior to sorting.

Instrument
Flow sorting was performed on a BD FACSAriaTM Fusion instrument

Software
FlowJoV10.4.1

Cell population abundance
Abundance of CD45 positive and negative fractions for droplet single sequencing were determined by cell counting post sort. Purity was checked indirectly by mini bulk RNAseq validation and cytospins for morphology. Additional purity checks for functional experiment (HSC differentiation culture) also included FACS index data and single cell RNA sequencing of sorted cells.

Gating strategy
As shown in Extended Data Figure 8, for all flow experiments, cells were gates based on FSC/SSC, live (DAPI negative set based on unstained cells from the sample sample) and single cells (FSC-H/FSC-A). For single cell sequencing, the ‘positive’ gate was set between the middle of positive and negative staining to the edge of plot, and ‘negative’ was set to everything else to ensure that all cells were accounted for. For validation experiments (mini bulk RNAseq, cytospins and culture sorts), gates were set over the bulk of the positive staining excluding the edges of staining. Our flow cytometry data adhere to the information standards for Flow cytometry (https://onlinelibrary.wiley.com/doi/pdf/10.1002/cyto.a.20623).

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.