Intrathymic dendritic cell-biased precursors promote human T cell lineage specification through IRF8-driven transmembrane TNF

Kai Ling Liang[1,2], Juliette Roels[1,11,15], Marieke Lavaert[1,12,15], Tom Putteman[1], Lena Boehme[1], Laurentijn Tilleman[3], Imke Velghe[1], Valentina Pegoretti[4], Inge Van de Walle[1,13], Stephanie Sontag[5,6], Jolien Vandewalle[7,8], Bart Vandekerckhove[1,2], Georges Leclercq[1,2], Pieter Van Vlierberghe[2,9], Claude Libert[7,8], Filip Van Nieuwerburgh[2,3], Roman Fischer[4,14], Roland E Kontermann[4], Klaus Pfizenmaier[4], Gina Doody[10], Martin Zenke[5,6], Tom Taghon[1,2]

[1]Department of Diagnostic Sciences, Ghent University, Ghent, Belgium.

[2]Cancer Research Institute Ghent, Ghent, Belgium.

[3]Laboratory of Pharmaceutical Biotechnology, Ghent University, Ghent, Belgium.

[4]Institute of Cell Biology and Immunology, University of Stuttgart, Stuttgart, Germany.

[5]Institute for Biomedical Engineering, Department of Cell Biology, RWTH Aachen University Medical School, Aachen, Germany.

[6]Helmholtz-Institute for Biomedical Engineering, RWTH Aachen University, Aachen, Germany.

[7]VIB Center for Inflammation Research (IRC), Technologiepark, Ghent, Belgium.

[8]Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium.

[9]Department of Biomolecular Medicine, Ghent University, Ghent, Belgium.

[10]Leeds Institute of Medical Research, University of Leeds, Leeds, UK.

[11]Present address: Genentech Inc., South San Francisco, CA, USA.

[12]Present address: Laboratory of Genome Integrity, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA.

[13]Present address: argenx BVBA, Zwijnaarde, Belgium.

[14]Present address: BioNtech, Mainz, Germany.

[15]These authors contributed equally: Juliette Roels, Marieke Lavaert.

Corresponding author:

Tom Taghon

Department of Diagnostic Sciences

Ghent University

C. Heymanslaan 10

MRB2, Entrance 38

9000 Ghent, Belgium

+32 (0) 9 332 01 33

tom.taghon@ugent.be

ABSTRACT

The cross talk between thymocytes and thymic stromal cells is fundamental for T cell development. In humans, intrathymic development of dendritic cells is evident but its physiological significance is unknown. Here, we showed that dendritic cell-biased precursors depended on the expression of the transcription factor Interferon Regulatory Factor 8 (IRF8) to express the membrane-bound precursor form of the cytokine Tumor Necrosis Factor (tmTNF) in order to promote differentiation of thymus seeding hematopoietic progenitors into T-lineage specified precursors through activation of the TNF receptor 2 (TNFR2) instead of TNFR1. *In vitro* recapitulation of TNFR2 signaling by providing low density tmTNF or a selective TNFR2 agonist enhanced the generation of human T cell precursors. Our study shows that, in addition to mediating thymocyte selection and maturation, dendritic cells function as a hematopoietic stromal support for the early stages of human T cell development and provide proof-of-concept that selective targeting of TNFR2 can enhance the *in vitro* generation of T cell precursors for clinical application.

KEYWORDS

human thymus, dendritic cell-biased precursors, T cell precursors, IRF8, Notch signalling, transmembrane TNF, TNFR2, hematopoietic stem and progenitor cells, in vitro T cell development, interferon signalling

T cell development occurs in the thymus, which is constantly seeded by bone marrow-derived hematopoietic progenitors. These thymus seeding progenitors (TSPs) undergo stepwise differentiation and eventual selection to generate a diverse and self-tolerant T cell repertoire that responds to foreign antigens. During this developmental process, cells migrate to distinct thymic microenvironments to receive appropriate site- and stage-specific signals through cellular contact with stromal cells. Non-hematopoietic stromal cells comprise mostly thymic epithelial cells (TECs) that constitute the thymic architecture. While TECs regulate T cell development throughout early and late stages, stromal cells of hematopoietic origin have only been described to mediate selection of developing T cells during final maturation. In human, these include thymus-residing dendritic cells (DCs) and B cells[1,2,3]. Recent studies provide compelling evidence of human in situ intrathymic development of dendritic but not B cells[4,5,6]. However, it remains unclear if these thymic DCs develop in a distinct, non T-lineage supporting thymic niche from multipotent TSPs in which T-lineage differentiation has not yet been initiated[6] or whether they can share an early developmental program with T cells[4,5]. Also the physiological relevance of the disparity in intrathymic DC vs B cell development is unclear given their common role in establishing T cell tolerance.

During early T cell development, TECs provide Notch ligands that induce T-lineage specification in TSPs to generate early T cell precursors (ETPs) and also produce cytokines such as interleukin-7 (IL-7) to support the survival and proliferation of immature thymocytes[7]. Recapitulation of these signals enables *in vitro* modelling and studying of T cell development[8,9]. However, a role for hematopoietic stromal cells in these early T-developmental stages has not yet been illustrated. Supplementation of TNF in a soluble form (sTNF) to *in vitro* culture systems temporarily and dose-dependently enhances the human T cell precursor generation from hematopoietic stem and progenitor cells (HSPCs)[10,11,12,13]. Physiologically, TNF is synthesized as a membrane-bound precursor (transmembrane TNF, tmTNF) that can be cleaved

to yield sTNF[14]. The production of TNF is weakly correlated with its gene expression due to post-transcriptional and translational regulation[15]. Although both tmTNF and sTNF are biologically active, the former is predominantly expressed[16]. At present, it is unclear if TNF is physiologically produced by thymic stromal cells and how the signal is transmitted to differentiating HSPCs. A comprehensive understanding of physiological signals provided by thymic stromal cells is important to unleash the full potential of *in vitro* T cell development for therapeutic application.

Based on the difference in developmental origin of thymus-residing DC and B cells, here we explored the physiological relevance of human in situ intrathymic DC development and found that DCs not only are important for mediating selection of thymocytes during the final maturation stages, but that immature DC-biased progenitors also provide stromal support for early T cell progenitors through IRF8-dependent tmTNF expression that activates TNFR2 signalling on immature thymocytes..

RESULTS

Notch-driven IRF8 expression marks T- and DC-lineage priming

Single-cell RNA sequencing (scRNA-seq) of *ex vivo* $CD34^+$ postnatal thymocytes previously identified two distinct subsets of TSPs[4]. Using the STEMNET algorithm, $CD34^+CD44^+CD10^+CD7^-$ TSP1s were predicted to be the canonical human T cell precursor, while $CD34^+CD44^+CD10^-CD7^+$ TSP2s that express *IRF8* were predicted to support both T and DC development, presumably through intrathymic expansion of a hematopoietic progenitor cell (HPC) population (Extended Data Fig. 1a). The transcription factor IRF8 represents a marker for a human DC-lineage development[17]. Based on the STEMNET prediction and using pseudotime analysis[4], we observed a gradual increase in *IRF8* expression along the differentiation trajectory of TSP2s towards pDCs via a granulocyte-macrophage progenitor (GMP) $IRF8^{hi}$ subset (Fig. 1a), consistent with the projection that TSP2s would differentiate along the DC lineage trajectory. Consistent with previous findings that the earliest human and murine thymic progenitors are transcriptionally distinct[4,5], IRF8 protein was not detected in immature murine thymocytes (Extended Data Fig. 1b,c). Thus, IRF8 expression in early T cell development is a distinct feature in humans.

To validate the previously annotated immature human thymocyte populations[4] phenotypically, we divided *ex vivo* $lin(lineage)^-CD4^-CD34^+$ thymocytes into four subsets based on expression of IRF8 and CD1a, a marker for human T-lineage commitment[18] (Fig. 1b,c), and further examined the expression of selected precursor (CD34, CD44, CD7, PU.1), T-lineage (CD5, CD127 (IL-7 receptor α chain), cytoplasmic CD3 (cyCD3), GATA3) and DC-lineage (HLA-DR, CD123, CD135, CD117) associated surface and intracellular markers on these subsets (Fig. 1d). This analysis revealed that the $IRF8^{lo}CD1a^-$ subset corresponded to the previously annotated TSP2 and HPC populations that both express high PU.1 levels which indicates multipotency. Consistent with the scRNA-seq analysis, these $lin^-CD4^-CD34^+CD123^{lo}CD1a^-$

thymocytes expressed markers representative of both T lineage (the transcription factor GATA3 and cyCD3) and DC lineage (CD44, CD123)[4,6,19,20]. On the other hand, the IRF8^{hi}CD1a^{-} subset, which corresponded to the annotated IRF8^{hi} GMP population, only expressed DC-lineage markers (CD44, HLA-DR and CD123), whereas the IRF8^{-}CD1a$^{-/+}$ subsets only expressed T-lineage markers (including CD5, CD127, GATA3 and cyCD3) (Fig. 1d,e). Importantly, overlay of these four immature thymocyte subsets (Extended Data Fig. 1d) revealed that the IRF8^{lo}CD1a^{-} subset co-expressed low levels of the T-lineage transcription factor GATA3 (Fig. 1f) and was thereby positioned at the bifurcation of the T- and DC-lineages. Hence, low IRF8 expression in CD34^{hi} immature thymocytes marked T- and DC-lineage priming rather than specification towards one of these lineage cell fates.

To validate the developmental potential of these subsets, we sorted *ex vivo* lin^{-}CD4^{-}CD34^{+} thymocytes based on expression of CD123, a surrogate marker for IRF8 expression[21,22] and CD1a expression into lin^{-}CD4^{-}CD34^{+}CD123^{hi}CD1a^{-}, lin^{-}CD4^{-}CD34^{+}CD123^{lo}CD1a^{-}, lin^{-}CD4^{-}CD34^{+}CD123^{-}CD1a^{-} and lin^{-}CD4^{-}CD34^{+}CD123^{-}CD1a^{+} subsets (Extended Data Fig. 1e-g), and co-cultured them on OP9-DLL4 stromal cells to study T-lineage potential and on OP9 cells to track DC development. We confirmed that CD123 tracked IRF8 expression in human CD34^{+} thymocytes (Extended Data Fig. 1g). The CD123^{lo}CD1a^{-} subset had low expression of IRF8 but higher expression of CD34 compared to the other populations being studied (Fig. 1d and Extended Data Fig. 1g), which matched with the IRF8^{lo}CD1a^{-} subset. Consistent with its bi-phenotypic profile and high CD34 expression, the CD123^{lo}(IRF8^{lo})CD1a^{-} subset efficiently differentiated into both CD7^{+}CD5^{hi} T cell precursors on OP9-DLL4 (Fig. 1g,h) and HLA-DR^{+}CD123^{+} plasmacytoid DCs (pDCs) and HLA-DR^{+}CD1c^{+} conventional DCs (cDCs) on OP9 (Fig. 1i,j). The CD123^{hi}(IRF8^{hi})CD1a^{-} subset, which is relatively more mature (Fig. 1d and Extended Data Fig. 1g), generated only negligible amounts of CD7^{+}CD5^{+} T cell precursors (Fig. 1g,h) and predominantly gave rise to pDCs (Fig. 1i,j), consistent with reports that human

pDC development is IRF8^{hi}-dependent whereas cDC development, depending on the subsets, requires low or high IRF8 expression[21]. In contrast, the CD123^{-}(IRF8^{-})CD1a^{-} and CD123^{-}(IRF8^{-})CD1a^{+} subsets did not develop into pDCs and cDCs (Fig. 1i,j), consistent with their reduced CD44 expression (Fig. 1d) as they undergo T-lineage commitment[20,23]. Overall, these observations indicated that the CD123^{lo}(IRF8^{lo})CD1a^{-} subset, corresponding to the TSP2 and HPC populations, possessed T and DC potential. To further investigate if T cells and DCs have a common developmental origin in the human thymus, we performed TCR rearrangement analysis. In humans, *Dδ2-Dδ3* rearrangements within the *TCR-δ* locus occur first, within the CD34^{+}CD7^{lo}CD5$^{-/lo}$CD1a^{-} thymic progenitors[24]. We detected comparable frequencies of *Dδ2-Dδ3* rearrangements in sorted *ex vivo* CD34^{+}CD123^{lo}(IRF8^{lo})CD1a^{-} and CD34^{+}CD123^{hi}(IRF8^{hi})CD1a^{-} subsets as in thymic cDCs and thymic pDCs (Fig. 1k), indicating that human thymic DCs originated from a thymic precursor that has already initiated TCR rearrangements and thus received T-lineage inductive signals.

IRF8^{lo} TSP2 and HPC populations also express CD7, a Notch target during early human T cell development[4,25]. To investigate if Notch signalling was permissive for induction of IRF8 expression in TSPs, we exposed human cord blood-derived CD34^{+}lin^{-} HSPCs (CB HSPCs hereafter) to Notch ligands. *IRF8* expression was only upregulated by DLL1-, DLL4- and JAG2-mediated activation of Notch signalling (Fig. 2a), consistent with the potential of these ligands, but not of JAG1, to induce human T-lineage specification[25]. Nevertheless, further and sustained IRF8 expression during intrathymic DC development at later stages could be less Notch-dependent[6]. IRF8 protein was significantly induced in CB HSPCs that overexpressed ICN1, a constitutively active form of Notch 1 (Fig. 2b,c). Immunophenotyping indicated that IRF8^{+} ICN1-transduced CB HSPCs displayed stronger expression of CD10, which is expressed by the HPC, but not the TSP2 population[4,13], and of CD127, compared to control- or IRF8^{-} ICN1-transduced CB HSPCs (Fig. 2d). Although IRF8 expression is promoted by Notch

signalling in human TSPs, analogous to murine macrophage development[26], IRF8 expression is gradually silenced in the subsequent T cell developmental stages[4]. Continuous high expression of IRF8 inhibited the development of $CD7^{+}CD5^{hi}CD1a^{+}$ T cell lineage committed precursors (Fig. 2e-g). GATA3 is a driver of human T-lineage commitment that restrains Notch signalling[20]. Because IRF8 was positively regulated by Notch signalling (Fig. 2a-d) and intrathymic IRF8 expression decreased with concomitant increased GATA3 expression (Fig. 2h), we tested whether silencing of IRF8 was GATA3-mediated. Enforced GATA3 expression in CB HSPCs significantly downregulated endogenous expression of IRF8 RNA and protein (Fig. 2i-k), an effect reversed upon *GATA3* knockdown (Fig. 2l). Thus, although IRF8 expression was permissive for T-lineage specification even at high levels, its silencing by GATA3 was mandatory to drive T-lineage commitment and fine-tuning of IRF8 expression was critical to discriminate between T- and DC-lineage development.

Low IRF8 activity promotes early development of T and DC lineages

To examine if low IRF8 expression promoted early T cell development, we transduced CB HSPCs with an empty control or IRF8-ERT2 fusion protein-encoding virus in which IRF8 transcriptional activity is regulated through tamoxifen-dependent migration to the nucleus (hereafter $HSPC^{IRF8\text{-}ERT2}$) (Extended Data Fig. 2a). At day 7 of OP9-DLL4 co-culture with 50 nM of 4 hydroxytamoxifen (4-OHT), low nuclear IRF8-ERT2 increased the generation of $CD7^{+}CD5^{+}$ T cell precursors compared to in the absence of 4-OHT or control transduced CB HSPCs (Fig. 3a,b). However, with higher doses of 4-OHT (100-300 nM), the T cell lineage promoting effect was gradually lost and a population of $CD34^{+}CD7^{+}CD5^{-}$ cells with gradually increased expression of CD123 developed (Fig. 3a,c), suggesting that low IRF8 expression was beneficial for T-lineage development, whereas high levels skewed differentiation to the DC lineage. To delineate the underlying mechanism, we sorted $CD7^{+}$ cells at day 4 of OP9-DLL4

culture for bulk RNA-seq. Although intracellular expression of IRF8-ERT2 was detected without 4-OHT treatment, $HSPC^{IRF8\text{-}ERT2}$ cells without 4-OHT had a similar transcriptome compared to control transduced cells (Extended Data Fig. 2b,c). Genes associated with interferon (IFN) signalling were significantly downregulated in 50 nM 4-OHT-treated $HSPC^{IRF8\text{-}ERT2}$ compared to control transduced cells (Fig. 3d and Extended Data Fig. 2d), suggesting that inhibition of IFN signalling was important for T-lineage differentiation. Consistently, UCell indicated that the IFN-related gene signature from the bulk RNA-seq analysis was highly enriched in TSPs, but gradually downregulated as they differentiated to become T-lineage specified and committed (Fig. 3e and Extended Data Fig. 2f). Notch target genes such as *CD3E* and *DTX1* were consistently upregulated in 50 nM 4-OHT-treated $HSPC^{IRF8\text{-}ERT2}$ compared to control transduced cells, although not statistically significant[20] (Extended Data Fig. 2e), while *TNF* was significantly upregulated (Fig. 3d). Using complementary ATAC-seq, we found that 92% of the significant changes in chromatin accessibility regions of control-transduced T-lineage specified $CD7^+$ cells compared to the more immature, non-specified $CD34^+CD7^-$ cells were shared by 50 nM 4-OHT-treated IRF8-ERT2-transduced $CD7^+$ cells, which also had more unique changes (Fig. 3f). Transcription factor motif analyses showed that those unique chromatin accessibility changes mostly harboured binding sites for ETS (opened regions) or GATA (closed regions) transcription factors (Extended Data Fig. 2g,h). The 50 nM 4-OHT-treated IRF8-ERT2-transduced $CD7^+$ cells appeared to develop further along the T-lineage compared to the $CD34^+CD7^-$ cells from these cultures as a *CEBPE* enhancer, which is essential for granulocytic differentiation, was significantly closed in these cells[27] (Fig. 3g). We detected significant opening of the *TNF* core promoter in 50 nM 4-OHT-treated IRF8-ERT2-transduced $CD7^+$ cells compared to in the $CD34^+CD7^-$ cells (Fig. 3g). TNF signalling through the TNF receptor 1 (TNFR1) and 2 (TNFR2) mediates both common and receptor-specific downstream signalling events[28]. To

clarify how *TNF* expression promoted human T cell precursor generation, we characterized the expression of TNF and TNFR on different subsets of $CD7^+$ cells. Surface expression of TNFR1 was barely detected (Extended Data Fig. 2i,j). In contrast, $CD7^+CD123^+$ progenitor cells co-expressed TNFR2 and tmTNF, while $CD7^+CD5^+$ T cell precursors only expressed TNFR2 (Fig. 3h,i). Our results indicate that low expression of IRF8 in Notch-stimulated HSPCs induces TNFR2 expression in CD34+CD7+CD123- precursors and induces both TNFR2 and tmTNF expression specifically in $CD34^+CD7^+CD123^+$ cells.

$CD34^+CD123^+IRF8^+tmTNF^+$ cells promote T-lineage specification

Using previously characterised wild-type and IRF8-deficient human induced pluripotent stem cells (iPSCs)[29], we next studied the impact of IRF8 loss on T cell development in the embryonic mesodermal organoid (EMO) system[30]. IRF8, which is dispensable for the development of $CD45^+CD34^+$ hematopoietic progenitors[29], was also dispensable for iPSCs to develop into embryonic mesodermal progenitors (EMPs) (Extended Data Fig. 3a,b) that are used to initiate the EMO cultures. *IRF8*$^{-/-}$ EMPs differentiated into $CD7^+CD5^+$ T cell precursors and $CD4^+CD8b^+$ double positive (DP) thymocytes similar as the wild-type *IRF8*$^{+/+}$ control EMPs (Fig. 4a,b), supporting the hypothesis that IRF8 might indirectly promote early T cell development[4]. Immunophenotyping (at day 14 after initiation of the EMOs), immediately after hematopoietic induction and prior to T-lineage differentiation, indicated that *IRF8*$^{-/-}$ EMPs were severely impaired to differentiate into a subset of $CD123^+CD127^+TNFR2^+$ HPCs compared to *IRF8*$^{+/+}$ control EMPs (Fig. 4c,d and Extended Data Fig. 3c). Wild-type $CD123^+CD127^+TNFR2^+$ HPCs expressed IRF8 and tmTNF (Fig. 4e**)**, while the few $CD123^+CD127^+TNFR2^+$ HPCs in the *IRF8*$^{-/-}$ EMOs had impaired tmTNF expression **(**Fig. 4f,g**).** Although cleavage of tmTNF could give rise to sTNF[14], we did not detect sTNF in the EMOs (Extended Data Fig. 3d-f) and conditioned medium from EMOs initiated with wild-type

IRF8$^{+/+}$ EMPs could not rescue the development of CD123^{+}CD127^{+}TNFR2^{+} HPCs from *IRF8*$^{-/-}$ EMPs (Extended Data Fig. 3g,h). Next, we sorted the CD45^{+}CD34^{+}CD123^{+}TNFR2^{+} subset for limiting dilution co-culture analysis, without staining of CD127 to avoid blockade of its function during development[13]. When cultured in T-stimulating conditions on OP9-DLL4-7FS stromal cells, these progenitors showed a higher potential to differentiate into HLA-DR^{+}CD123^{+} DC-lineage cells compared to in CD7^{+}CD5^{+} and CD4^{+}CD8b^{+} T cell precursors (Fig. 4h,i). To investigate if IRF8-dependent HLA-A2^{+}CD123^{+}CD127^{+}tmTNF^{+} progenitors could promote T cell precursor generation from HLA-A2^{-} HSPCs in a non-cell-autonomous manner, we sorted these iPSC-derived CD123^{+} cells and added 1,450 of these cells per artificial thymic organoid (ATO)[9]. Compared to the control ATOs to which no iPSC-derived CD123^{+} cells were added, HSPCs gave rise to more CD7^{+}CD5^{+} T cell precursors in the presence of HLA-A2^{+}CD123^{+}CD127^{+}tmTNF^{+} precursors (Fig. 4j,k and Extended Data Fig. 3i-l) and these T cell precursors were more mature, as evidenced by expression of CD1a (Fig. 4l)[20]. Collectively, our data showed that IRF8 promoted human T cell precursor generation indirectly through cellular crosstalk and through the development of DC-biased tmTNF-expressing CD123^{+}CD127^{+}TNFR2^{+} progenitors.

TNFR2 is induced during human T-lineage specification

We observed that TSP2 and HPC-annotated *ex vivo* immature CD7^{+}CD123^{lo}(IRF8^{lo})CD1a^{-} thymocytes had the highest expression of tmTNF compared to the other CD34^{+} thymocyte subsets (Fig. 5a,b and Extended Data Fig. 4a). Compared to TSP2s and HPCs, CD34^{+}CD7^{+}CD123^{-}CD1a^{-} ETP and T-lineage specified thymocytes (subset 4) displayed similar expression of TNFR2 and CD127, but lower tmTNF expression (Fig. 5a,b and Extended Data Fig. 4a). The T-lineage committed CD34^{+}CD1a^{+} thymocytes (subset 5) had the lowest expression of tmTNF, but the highest expression of CD127 (Fig. 5a,b and Extended Data Fig.

4a). Because TNF signalling can be modulated by changes in TNFR1 and TNFR2 expression during human aging[31], we used the ATO system[9] to characterize the kinetic expression of TNFR1 and TNFR2 during early T cell development on differentiating HSPCs derived from three human ontogenetic stages. At day 2 of culture, only cord blood-derived HSPCs expressed detectable amounts of TNFR1 (Extended Data Fig. 4b) but this was gradually downregulated as HSPCs differentiated along the T-cell lineage (Extended Data Fig. 4b). TNFR1 expression was also hardly detected on the differentiating HSPCs in OP9-DLL4 co-culture assay at day 7 (Extended Data Fig. 2i,j) and on *ex vivo* immature $CD34^+$ thymocytes (Fig. 5b). In contrast, TNFR2 expression was induced on buffy coat-derived HSPCs or maintained on CB and foetal liver-derived HSPCS (Extended Data Fig. 4c,d), and transiently co-expressed with the Notch-dependent CD7 marker[25] (Fig. 5c,d), suggesting that TNF signalling was mediated by TNFR2 during early human T cell development.. Expression of CD5 on $TNFR2^+CD7^+$ cells suggested their T lineage identity (Fig. 5e), which was corroborated by the observation that development of $TNFR2^+CD7^+$ cells was impaired in ATO cultures without IL-7 (Fig. 5f,g), resulting in expression of CD123 instead of CD5 (Fig. 5h). Given that CD7 expression on hematopoietic progenitors marks loss of myeloid and erythroid potential[32,33], our data suggested that such lineage restriction might occur earlier, as TNFR2 expression precedes CD7 induction. In an ATO system spiked with MS5 stromal cells that expressed both human DLL4 and human tmTNF (MS5-DLL4-tmTNF) (Extended Data Fig. 5), $TNFR2^+$ HSPCs had higher T cell potential compared to $TNFR2^-$ HSPCs (Fig. 5i-k). $TNFR2^+$ HSPCs also gave rise to significantly less granulocytes compared to $TNFR2^-$ HSPCs when cultured in myeloid-stimulating conditions on MS5 stromal cells (Fig. 5l,m). Based on the expression kinetics of TNFR2, our results indicate that TNFR2-mediated TNF signalling plays an early role, prior or in parallel to Notch signalling, to steer the development of TSPs to T-lineage specified progenitors.

TNFR2 targeting enhances human T cell precursor generation

Because robust activation of TNFR2 requires binding of tmTNF instead of sTNF[34,35], we tested whether activation of TNFR2-dependent TNF signalling maximized human T cell precursor generation *in vitro*. We used MS5-DLL4-tmTNF stromal cells to mimic the physiological tmTNF signal provided by $CD123^+CD127^+$ DC-biased progenitors in the conventional ATO system[9]. Unlike sTNF, tmTNF can activate both TNFR1 and TNFR2 upon binding[34,35]. To test whether a low tmTNF density would allow preferential TNFR2 targeting on differentiating HSPCs while minimizing TNFR1 activation, which is normally downregulated during T-lineage development, we assembled ATOs using different MS5-DLL4-tmTNF/MS5-DLL4 ratios and examined human $CD7^+CD5^+$ T cell precursor generation. At day 10 of culture, cellular output was similar in ATO in which MS5-DLL4-tmTNF represented 1 to 100% of the total MS5 cells compared to control ATO without MS5-DLL4-tmTNF stromal cells (Fig. 6a). However, MS5-DLL4-tmTNF, regardless of its frequency in the mix, increased the frequency and yield of $CD7^+CD5^+$ T cell precursors compared to in its absence (Fig. 6b-d). Moreover, we observed a significant linear trend of an inverse correlation between the MS5-DLL4-tmTNF density and the cellular pool of undifferentiated $CD34^+$ HSPCs (Fig. 6b-d). A low tmTNF density (1%) expanded $CD7^+CD5^+$ T cell precursors without skewing the differentiation of HSPCs over proliferation (Fig 6d), and the generated $CD7^+CD5^+CD1a^+$ T cell precursors displayed the lowest HLA-DR expression, similar to in the control ATO that lacks MS5-DLL4-tmTNF (Fig. 6e,f).. Because upregulation of HLA-DR is a unique feature of TNFR1-specific signalling[36], these observations indicated that provision of tmTNF signal *in vitro* at the 1% MS5-DLL4-tmTNF density selectively targeted TNFR2 and maximized the T cell precursor generation per HSPCs. Consistently, the frequency and yield of T cell precursors also increased in an ATO in the absence of MS5-DLL4-tmTNF but with supplementation of EHD2-

317 scTNF$_{R2}$[37], a TNF mutein and TNFR2-selective agonist, compared to the control that lacked
318 both the agonist and MS5-DLL4-tmTNF (Fig. 6g,h and Extended Data Fig. 6). Thus, these
319 result show that selective TNFR2 activation promotes early T cell development.

320

321 **sTNF/TNFR1-specific signalling accelerates T lineage differentiation**

322 sTNF has been used to enhance human T cell precursor generation[10,11,12] and the sTNF-TNFR1
323 signaling was shown to regulate human HSPCs fate by promoting their differentiation but not
324 their self-renewal[38]. To test whether the reported promoting effect of sTNF on human T cell
325 precursor generation resulted from the accelerated differentiation of HSPCs at the expense of
326 their maintenance, we used an ATO system with only MS5-DLL4 in which T cell development
327 is supported by a minimum number of cytokines at minimal concentration (IL-7 and FLT3-L
328 at 5 ng/mL)[9] (Extended Data Fig. 7a). This allowed to determine the effect of sTNF on human
329 T cell precursor generation without interference from cytokines such as SCF and
330 thrombopoietin (TPO), which are known to stimulate HSPC proliferation and are used in other
331 *in vitro* systems[10,11,12,39]. On day 10 of culture, we observed that sTNF at 0.25, 5 and 100 ng/mL
332 did not improve the total ATO cell yield compared to in the control ATOs that lacked sTNF
333 (Extended Data Fig. 7b). Furthermore, high dose of sTNF (100 ng/mL) significantly decreased
334 the total yield compared to the control without sTNF (Extended Data Fig. 7b). Increasing doses
335 of sTNF were linearly correlated with the depletion of undifferentiated CD34$^+$ HSPCs, but
336 inversely correlated with the yield of CD7$^+$CD5$^+$ T cell precursors (Extended Data Fig. 7c-e).
337 Only the lowest tested dose (0.25 ng/mL) of sTNF significantly increased T cell precursor
338 generation because the CD34$^+$ HSPC pool was depleted to the least extent compared to the
339 higher doses of 5 and 100 ng/mL sTNF (Extended Data Fig. 7e).. Overall, this indicated that,
340 in the presence of Notch signalling, HSPCs were dose-dependently steered by sTNF to favour
341 T-lineage differentiation instead of proliferation and that the previously reported positive effect

of high dosage of sTNF on human T cell precursor expansion was inadvertently contributed by the activity of other cytokines that counteract the HSPC-depleting effect of sTNF and that were used in those systems[10,11,12]. Furthermore, sTNF dose-dependently increased HLA-DR expression, a unique feature of TNFR1-specific signalling[36], in CD1a^{+} T-lineage committed precursors (Extended Data Fig. 7f,g), which physiologically have minimal expression of this marker[20]. Because activation of TNFR1 and TNFR2 can induce both common and receptor-specific downstream signalling[28], our data suggested that low-dose sTNF increased T cell precursor generation by circumventing activation of TNFR1-specific downstream signalling that could lead to aberrant HLA-DR expression and depletion of HSPCs, while retaining the activation of downstream signalling common to TNFR1 and TNFR2, which promotes early T cell development in humans.

TNF downregulates IFN-related genes in T-cell precursors

To better understand how TNF signalling promoted early T cell development, we performed single-cell RNA sequencing (scRNA-seq) on CB HSPCs differentiated in MS5-DLL4 ATOs supplemented with 0.25 ng/mL sTNF and in MS5-DLL4 ATOs with 1% MS5-DLL4-tmTNF which recapitulates physiological TNFR2 activation to unravel the global impact of TNF signalling compared to on CB HSPCs differentiated in control MS5-DLL4 ATO[9]s. Following quality control and dimensionality reduction analyses, 26 clusters labelled cluster 0 to cluster 25 were identified (Fig. 7a), containing cells derived from all conditions (Extended Data Fig. 8a) and at different cell cycle phases (Extended Data Fig. 8b). Annotation of these clusters based on cell-type specific gene markers allowed to depict the heterogeneity present within the differentiating HSPCs, despite being cultured in T cell-stimulating conditions (Fig. 7b). Nevertheless, clusters that annotated as T cell progenitors and that express T-lineage genes such as *RAG2*, *CD1E* and *BCL11B* comprised the biggest population among the differentiating cells[5]

(Fig. 7b,c). Consistent with the effect of TNF signalling on early T cell development, 45.6% and 41.5% of the cells labelled as T-progenitors were derived from sTNF-treated (0.25 ng/mL) and MS5-DLL4-tmTNF (1% density) ATOs, respectively (Fig. 7c). scRNA-seq analyses also identified that sTNF and tmTNF had differential impacts on the development of other hematopoietic populations. For example, 62.3% of the cells labelled as macrophages were derived from 0.25 ng/mL sTNF-treated ATOs, whereas 69.5% of the cells labelled as mast cells were derived from MS5-DLL4-tmTNF (1% density) ATOs (Fig. 7c), suggesting that sTNF-TNFR1 or tmTNF-TNFR2 signalling in differentiating HSPCs resulted in receptor-specific downstream signalling., We had observed that IRF8 induced tmTNF in DC-biased precursors and also reduced expression of IFN-related genes in the IRF8-ERT2 differentiated T cell precursors. Therefore, we analysed the IFN-related gene signature in this scRNAseq dataset and observed that this gene signature was highly enriched in the lymphoid progenitors and in the DC populations compared to in T-progenitors (Fig. 7d and Extended Data Fig. 8c). More than 70% of the lymphoid progenitors (clusters 4 and 15) were derived from the control, non TNF-stimulated ATOs since T-lineage specification was promoted in both TNF-activated ATOs (0.25 ng/mL sTNF-treated ATOs and MS5-DLL4-tmTNF (1% density) ATOs, Extended Data Fig. 8d). The IFN-related gene expression was downregulated in the TNF-activated lymphoid progenitors from 0.25 ng/mL sTNF-treated ATOs and MS5-DLL4-tmTNF (1% density) ATOs compared to in lymphoid progenitors from the MS5-DLL4 control ATOs (Fig. 7e). The expression of these genes was gradually downregulated as TSPs differentiated along the T-lineage (Extended Data Fig. 8e), but was highly enriched in intrathymic DC intermediate populations[4]. These data indicated that, in the presence of Notch signalling, activated TNF signalling helped steer HSPCs towards T-lineage differentiation by inhibiting IFN signalling, which would otherwise support DC-lineage development.

Continuous exposure of HSPCs to sTNF was reported to inhibit the development of DP thymocytes[10]. To investigate if physiological tmTNF-TNFR2 stimulation resulted in the generation of T cell precursors with enhanced T cell maturation potential compared to sTNF-TNFR1 activated cells, we sorted 0.25ng/mL sTNF and 1%tmTNF stimulated $CD7^+$ progenitors after 8 days of ATO culture and reaggregated them in secondary MS5-DLL4 ATOs (Extended Data Fig. 8f). In the absence of further TNF stimulus, sTNF-exposed $CD7^+$ progenitors generated, on average and after 13 days of secondary culture, 2-fold less DP thymocytes compared to control, non-TNF exposed $CD7^+$ progenitors (Fig. 7f,g and Extended Data Fig. 8g,h) although they had significantly higher outputs of $CD3^+TCR\alpha\beta^+$ and $CD3^+TCR\gamma\delta^+$ T cells (3,8- and 3,1-fold, respectively) after 25 days of secondary ATO culture (Fig. 7h-j and Extended Data Fig. 8i-l). In contrast, tmTNF-exposed $CD7^+$ progenitors (isolated after 8 days of ATO) produced a 2,4-fold higher output of $CD4^+CD8b^+$ DP thymocytes at day 13 of secondary ATO culture compared to control, non-TNF exposed $CD7^+$ progenitors (Fig. 7f,g and Extended Data Fig. 8g,h). At day 25 of secondary ATO culture, tmTNF-exposed $CD7^+$ progenitors yielded higher outputs of $CD3^+TCR\alpha\beta^+$ and $CD3^+TCR\gamma\delta^+$ T cells (8,3- and 4,3-fold, respectively, Fig. 7h-j and Extended Data Fig. 8i-l), indicating that tmTNF-TNFR2 signalling *in vitro* generates more T cell precursors, that yield more mature T cell progeny compared to induced sTNF-TNFR1 signalling.

DISCUSSION

Our study unravels the physiological significance of intrathymic DC development in supporting early human T cell development, thereby clarifying their in situ intrathymic development from TSPs.. We showed that the DC- and T-lineages were concurrently primed in TSPs that expressed low levels of IRF8. Consistent with the presence in the thymus of $CD34^+IRF8^{hi}$ DC precursors and mature pDCs and cDCs that display *TCRD* gene rearrangements, we showed

that TSP2- and HPC-annotated $CD34^{+}CD123^{lo}IRF8^{lo}$ human thymocytes were bi-phenotypic, had initiated *TCRD* rearrangements and had the potential to develop into T- and DC-lineage cells[4]. Physiologically, we found that IRF8 expression in human HSPCs was upregulated following Notch signalling though Notch ligands (DLL1, DLL4 and JAG2) that can induce human T-lineage specification[25]. Furthermore, although IRF8 was reported as a marker for human DC-lineage specification[17], we found that enforced expression of IRF8 in human HSPCs was permissive for T-lineage specification. Overall, our study indicates that activation of Notch signalling in human TSPs not only induced T cell development but also supported DC-lineage differentiation through the induction of IRF8 expression.

DLL1-dependent Notch signalling was shown to promote *in vitro* differentiation of cDCs from both mouse and human hematopoietic progenitors[40,41]. However, single-cell analysis of the earliest CD117-expressing murine thymic progenitors did not uncover a developmental trajectory of DC-lineage cells[5,42]. Although thymic DCs exist in the murine thymus, they appear to be derived from $CD117^{-}$ thymic progenitors that do not sustain T cell development at physiological steady state[43,44,45]. Thus, murine thymic DCs seem to have a separate developmental origin compared to T cells and we also did not detect IRF8 protein in these CD117-expressing murine thymocytes. The rationale for this absence of IRF8 expression and of the correlated developmental trajectory of the DC-lineage from the murine counterparts of human $CD34^{+}$ thymocytes is unclear. Nevertheless, this further highlights that the downstream Notch network during early T cell development is different in human compared to in mice, in addition to the known differences in Notch activation status[18]. Physiologically, we found that IRF8 expression was silenced by GATA3, which restrains Notch signalling to induce human T-lineage commitment[20]. Hence, the unique expression of intrathymic IRF8 might necessitate and explain the earlier peak in the kinetic expression profile of *GATA3* compared to other critical T-lineage transcription factors, such as *TCF7* and *BCL11B,* during the ETP and

specification stages of human T cell development and this results in altered dynamics of the activity of these factors compared to what has been described in mice[4,20,46,47].

To date, thymus-residing human DCs have been described to support thymocyte selection and maturation[2,3]. Our work demonstrated that intrathymic DC progenitors (TSP2 and HPC-annotated $CD34^+$ thymocytes) had an early role in promoting human T-lineage specification. Consistent with IRF8 expression being linked to the intrathymic DC potential of human T cell precursors, we could genetically demonstrate that the development of $CD123^+CD127^+$ DC-biased progenitors and their expression of tmTNF were IRF8-dependent. Our work also showed that these DC-biased progenitors and T cell precursors arose from differentiating human HSPCs that expressed TNFR2 in a Notch-stimulating microenvironment, thereby further consolidating that intrathymic DC and T cells share a common developmental origin. However, T cell precursors that were IRF8-independent did not express tmTNF, and their expansion via TNFR2 activation was instead mediated by cellular cross-talking with tmTNF-expressing DC-biased progenitors. Importantly, we clarified that tmTNF, instead of sTNF, was the physiological thymic signal that lead to activation of TNF signalling through TNFR2, instead of TNFR1, to ensure generation of developmentally competent human T cell precursors. sTNF was reported to enhance human T cell precursor generation *in vitro*[10,11,12,13]. However, we found that sTNF activated TNFR1-specific downstream signalling and lead to exhaustion of HSPCs and accelerated differentiation into T-lineage committed human precursors that displayed aberrant HLA-DR expression since *ex vivo* T-lineage committed thymocytes have minimal expression of this cell surface marker[36,38]. We showed that although sTNF-TNFR1 activation could partially mimic the physiological tmTNF-TNFR2 axis in promoting early T cell development, which might involve suppression of IFN signalling, TNFR2-specific downstream signalling events seem important to ensure that the generated T cell precursors are developmentally competent. Since HSPCs from foetal liver and CB display increased TNFR2

466 expression compared to adult HSPCs, the requirement for TNFR2-specific activation to ensure
467 efficient T cell development may explain the faster and more efficient T cell development from
468 foetal compared to adult HSPC sources[48,49,50]. TNFR2 is also known to uniquely mediate the
469 PI3K-AKT-mTOR pathway, which has been implicated in the development of DP
470 thymocytes[51,52] and the pathogenesis of T cell acute lymphoblastic leukaemia[28,53]. Hence,
471 further delineation of the downstream pathways of TNFR2-mediated physiological TNF
472 signalling that are involved in the development of human T cell precursors by multi-omics
473 approaches could improve our understanding of their malignant transformation during
474 leukemogenesis.

475 We demonstrated that selective targeting of TNFR2, by presenting tmTNF at low density or by
476 using a TNFR2-specific agonist, enhanced human T cell precursor generation. This provides a
477 proof-of-concept to apply selective targeting of TNFR2 to maximize the *in vitro* generation of
478 bona fide T cell precursors for clinical applications such as immune reconstitution and
479 immunotherapy. Currently, TNFR2 agonists have been in active development with the aim to
480 treat inflammatory and autoimmune diseases[28]. Future study is warranted to explore the
481 possibility of repurposing these agonists to unleash the full potential of *in vitro* T cell
482 development for therapeutic purpose.

483

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AUTHOR CONTRIBUTIONS

K.L.L. conceived the study, designed and performed experiments, analysed data and wrote the manuscript. J.R. analysed bulk ATAC-seq data. M.L. and T.P. analysed the previously published scRNA-seq data. T.P. and L.B. analysed the scRNA-seq data generated in this study. L.T. analysed bulk RNA-seq data. I.V. assisted to set up experiments. I.V.W. performed an experiment related to regulation of *IRF8* expression. J.V., B.V., G.L., P.V.V. and C.L. provided reagents. F.V.N provided expertise in ATAC- and RNA-seq. V.P., R.F., R.E.K. and K.P. provided TNFR2-selective TNF mutein (EHD2-scTNF$_{R2}$). G.D. provided *IRF8*-related constructs. S.S. and M.Z. provided *IRF8*$^{+/+}$ and *IRF8*$^{-/-}$ iPSCs. T.T. supervised the study,

designed experiments, and wrote the manuscript. All authors have seen, reviewed and approved the final version of the manuscript.

COMPETING INTERESTS

K.L.L. and T.T. have filed a PCT application (PCT/EP2022/063712: Generating T cell precursors via agonizing tumour necrosis factor receptor 2) with the European Patent Office on 20th May 2022. K.P., R.F. and R.E.K. are named inventors on a patent covering the TNFR2-specific agonist (EZH2-scTNF$_{R2}$). R.F. and R.E.K. received funding from Resano, a company which has licenced the technology to generate TNFR2-specific agonist. R.E.K. is a consultant for Immatics, Oncomatryx, Roche and SunRock . K.P. is a consultant for Oncomatryx, Resano and SunRock. The remaining authors declare no competing interests.

FIGURE LEGENDS

Fig. 1 | TSP2- and HPC-annotated CD34$^+$ human thymocytes express low levels of IRF8 and are bi-phenotypic and potent for T- and DC-lineages.

a, mRNA expression of *IRF8* and *IL3RA* for the previously annotated populations of human CD34$^+$ thymocytes[4]. The number of cells for each population is indicated above the violin plot. **b-d**, Flow cytometric gating of *ex vivo* lin$^-$CD4$^-$CD34$^+$ thymocytes (n=8) based on their expression level of IRF8 and CD1a (**b**), frequencies in a base-10 log scale (**c**) and their protein expression profiles (**d**) of the cellular subsets (n=8) identified in **b**. **e,** Heatmap illustrating the relative mRNA expression of the additional markers shown in **d** for the annotated CD34$^+$ thymocyte populations that correspond to the cellular subsets identified in **b**. **f**, Protein expression profile of lin$^-$CD4$^-$CD34$^+$ thymocytes for PU.1 and CD123 (n=2), coloured to display median fluorescence intensity of IRF8 (left) and GATA3 (right). The arrow head

indicates IRF8^{lo} cells at the bifurcation of T- and DC-lineages. **g,h**, Flow cytometry analysis to identify CD45^{+}CD7^{+}CD5^{hi} T cell precursors (*n*=3), derived from the OP9-DLL4 co-culture of the sorted subsets at day 7 (**g**), and their normalized absolute counts (**h**). **i,j**, Flow cytometry analysis to identify CD45^{+}HLA-DR^{+}CD1c^{+} cDCs and CD45^{+}HLA-DR^{+}CD123^{+} pDCs (*n*=3), derived from the OP9 co-culture of the sorted subsets at day 7 (**i**), and their normalized absolute counts (**j**). **k**, The relative frequency of *TCRD* rearrangement, in a base-10 log scale, detected in the sorted immature (*n*=2 except *n*=3 for CD123^{hi}CD1a^{-} subset) and mature (*n*=3 for cDCs, pDCs, B cells and αβ T cells) *ex vivo* hematopoietic cells. HL-60 is a promyelocytic leukaemia cell line (negative control). Data are representatives of eight (**b**), two (**d**,**f**) and three (**g**,**i**) independent experiments. Data are presented as mean±s.d. of eight (**c**), three (**h**,**j**) and one (**k**: CD123^{hi}CD1a^{-} subset and mature cells) independent experiments. Data are presented as mean of one experiment (**k**: immature cells except CD123^{hi}CD1a^{-} subset). Data are analysed by two-way ANOVA with Šídák's multiple comparisons test (**j**). *n*, biological replicates (**c**,**h**,**j**,**k**); exact *P* values are provided in the Source data.

Fig. 2 | IRF8 expression is induced during human T-lineage specification but is silenced in the subsequent commitment stage.

a, Relative mRNA expression of *IRF8* for cord blood HSPCs (*n*=2), co-cultured for 3 days on OP9 stromal cells that express green fluorescent protein (GFP) only (control) or different human Notch ligands. **b-d**, Flow cytometric analysis of IRF8 expression in sorted lin^{-} CD34^{+}GFP^{+} cord blood HSPCs (*n*=5) that were transduced with empty vector (control) or ICN1 (**b**). Frequency of IRF8^{+} cells (*n*=5) identified in **b** (**c**). Expression of CD10 and CD127 for the IRF8^{-} and IRF8^{+} fractions identified in **b** (**d**). **e-g**, Flow cytometric staining for CD45^{+}CD7^{+}CD5^{lo} and CD45^{+}CD7^{+}CD5^{hi} T cell precursors, generated from control and IRF8 transduced cord blood-derived HSPCs, in OP9-DLL4 co-culture (*n*=3) at day 14 (**e**). Frequency

563 of T cell precursors (*n*=3) identified in **e** (**f**) and their expression for CD1a (**g**). **h**, Flow
564 cytometric staining of IRF8 and GATA3 expression in *ex vivo* human thymic progenitors (*n*=8).
565 **i-k**, IRF8 expression, at mRNA (**i**: *n*=3) in a base-10 log scale and protein levels (**j,k**: *n*=4), in
566 sorted lin$^-$CD34$^+$GFP$^+$ HSPCs that overexpressed empty vector (control) and GATA3 at day 2
567 post transduction. **l**, mRNA expression of *IRF8* and *GATA3* in empty vector (control) and
568 *GATA3* short hairpin RNA (shRNA)-expressing HSPCs (*n*=3) at day 2 post transduction. Data
569 are representatives of two (**b**,**d**,**e**,**g**,**j**) and eight (**h**) independent experiments. Data are presented
570 as mean of two independent experiments (**a**). Data are presented as mean±s.d. of two
571 independent experiments (**c**,**f**,**i**,**k**,**l**). Data are analysed by two-tailed paired t test (**c**,**i**,**k**,**l**) and
572 two-way ANOVA with Šídák's multiple comparisons test (**f**). *n*, biological replicates
573 (**a**,**c**,**f**,**i**,**k**,**l**); exact *P* values are provided in the Source data.
574

575 **Fig. 3 | Low dose of active IRF8 promotes generation of tmTNF-expressing CD7$^+$CD123$^+$**
576 **progenitors and T cell precursors.**
577 **a-c**, Flow cytometric analyses of the transduced cells (*n*=4) treated without or with increasing
578 doses of 4-OHT at day 7 post OP9-DLL4 co-culture. T cell precursors were identified as
579 CD45$^+$GFP$^+$CD7$^+$CD5$^+$ whereas the CD45$^+$GFP$^+$CD7$^+$CD5$^-$ cellular fraction was examined
580 further to identify cells that co-express CD34 and CD123 (**a**). Absolute counts of CD7$^+$CD5$^+$ T
581 cell precursors (**b**) and CD7$^+$CD123$^+$ progenitors (**c**) identified in **a**. **d**, Bulk RNA-seq analysis
582 to determine IRF8-mediated changes in the transcriptional landscape of CD45$^+$GFP$^+$CD7$^+$
583 cells (*n*=3) at day 4 post OP9-DLL4 co-culture. Genes that were significantly downregulated
584 in the presence of low dose ofactive IRF8 constituted the IFN-related gene signature. **e**, IFN-
585 related gene signature was scored in the previously annotated populations of *ex vivo* CD34$^+$
586 thymocytes[4]. **f,g**, ATAC-seq (*n*=3) analysis to identify IRF8-mediated changes in the chromatin
587 accessibility landscape of CD45$^+$GFP$^+$CD34$^+$CD7$^-$ and CD45$^+$GFP$^+$CD7$^+$ cells at day 4 post

OP9-DLL4 co-culture (**f**). Genome browser view of ATAC-seq footprint around the *CEBPE* and *TNF* gene locus (**g**). **h,i**, Flow cytometric analysis (n=3) to examine the expression of tmTNF and TNF receptor 2 (TNFR2) during early T cell development at day 7 post OP9-DLL4 co-culture (**h**) and their expression patterns were quantified proportionally (**i**). Data are presented as mean±s.d. of three (**b**,**c**) and one (**i**) independent experiments. Data are representatives of three (**a**) and one (**h**) independent experiments. Data are analysed by one-way ANOVA with Dunnett's multiple comparisons test (**b,c**) and two-way ANOVA with Šídák's multiple comparisons test (**i**). *n*, biological replicates (**b**,**c**,**i**); exact *P* values are provided in the Source data.

Fig. 4 | IRF8-dependent DC-biased CD123$^+$CD127$^+$ progenitors express tmTNF and augment generation of T cell precursors via cellular crosstalk.

a,b, At day 14 post T cell differentiation of EMO cultures, CD7$^+$CD5$^+$ T cell precursors and the more mature CD4$^+$CD8b$^+$ subset were identified by flow cytometric analysis (**a**) and quantified in a base-10 log scale (**b**) (n=4 per genotype). **c,d**, Flow cytometric analysis to determine the impact of IRF8 loss on the generation (**c**) and cellular yield (**d**) of TNFR2$^+$CD123$^+$CD127$^+$ hematopoietic progenitors (n=8 per genotype). **e**, Characterization of IRF8 (left) and tmTNF (right) expression in CD123$^+$CD127$^+$ versus CD123$^-$CD127$^-$ cells within CD45$^+$CD34$^+$TNFR2$^+$ gated *IRF8*$^{+/+}$ precursors (n=4). **f,g**, Comparison (**g**) and quantification (**h**) of tmTNF expression in *IRF8*$^{+/+}$ versus *IRF8*$^{-/-}$ CD45$^+$CD34$^+$TNFR2$^+$CD123$^+$CD127$^+$ gated precursors (n=4 per genotype). **h,i**, 10, 25, 50 and 100 of iPSC-derived TNFR2$^+$CD123$^+$ hematopoietic progenitors were sorted for co-culture with OP9-DLL4-7FS stromal cells. At day 14, flow cytometric analysis were performed to identify cells of T- and DC-lineages (**h**), and lineage potential of the sorted cells was determined (**i**). **j-l**, At day 10 post ATO cultures, flow cytometric analyses to identify CD7$^+$CD5$^+$ T cell

precursors generated from cord blood-derived lin$^-$CD34$^+$CD38$^-$ HSPCs (*n*=3; HLA-A2$^-$) or iPSC-derived TNFR2$^+$CD123$^+$ hematopoietic progenitors (HLA-A2$^+$) (**j**). Relative quantification of the frequency and number of T cell precursors (*n*=3) generated from HLA-A2$^-$ HSPCs in the spiked-ATOs compared to the control (**k**) and flow cytometric analysis of their CD1a expression (**l**). Data are presented as mean±s.d. of one (**b**,**k**), three (**d**) and two (**g**) independent experiments. Data are representatives of one (**a**,**h**,**j**,**l**), three (**c**) and two (**e**,**f**) independent experiments. Data are analysed by two-tailed paired t test (**d**,**g**) and one sample two-tailed t test (**k**). CI, confidence interval; FC, fold change; *n*, pool of 2 EMOs (**b**,**d**,**g**) and biological replicates (**k**); exact *P* values are provided in the Source data.

Fig. 5 | TNFR2 expression precedes induction of CD7 during early human T cell development.

a,**b**, Flow cytometric analysis to identify 5 cellular subsets (labelled 1-5) of *ex vivo* lin$^-$CD4$^-$CD34$^+$ thymocytes (**a**). The developmental relationship (left) of all cellular subsets identified in **a** and their expression of TNFR1, TNFR2, tmTNF and CD127 (right; *n*=4) (**b**). **c-e**, Flow cytometric analysis of ATO cultures (CD45$^+$ gated) that were assembled using HSPCs derived from human fetal liver (CD34^{hi}CD45$^+$; *n*=2), cord blood (lin$^-$CD34$^+$CD38$^-$; *n*=2) or adult buffy coats (lin$^-$CD34$^+$; *n*=4) (**c**). Quantification of the proportional changes in the 4 cellular subsets shown in **c** throughout the culture period (**d**). Expression of CD5 on the 4 cellular subsets of buffy coat-ATOs shown in **c** at day 10 post culture (**e**). **f-h**, At day 10 post culture, cord blood-ATOs (lin$^-$CD34$^+$CD38$^-$; *n*=3) supplemented with and without IL-7 were immunophenotyped for the expression of TNFR2 and CD7 (**f**), and proportional changes in the 4 cellular subsets were quantified (**g**). The TNFR2$^+$CD7$^+$ subset was examined further for the expression of CD5 and CD123 (**h**). **i-m**, Expression of TNFR2 (left) and CD38 (right) on TNFR2$^-$ and TNFR2$^+$ fractions that were sorted from cord blood lin$^-$CD34$^+$ HSPCs (**i**).Flow cytometric analysis of

TNF-activated ATO cultures at day 10, that were assembled using TNFR2$^-$ and TNFR2$^+$ fractions (n=3), to identify CD7$^+$CD5$^+$ T cell precursors (**j**) and their absolute counts (**k**). Flow cytometric analysis of TNFR2$^-$ and TNFR2$^+$ fractions (n=3), that were co-cultured with MS5 stromal cells, to identify CD15$^+$CD14$^-$ granulocytes, CD15$^-$CD14$^+$ monocytes and CD34$^+$ immature progenitors (**l**), and their absolute counts (**m**). ATOs from each ontogenetic stage (data are presented as mean for fetal liver- and cord blood-ATOs, and as mean±s.d. for buffy coat-ATOs) were assembled and analysed independently (**d**). Data are presented as mean±s.d. (**b**,**g**,**k**,**m**) and representatives (**a**,**c**,**e-f**,**h-j**,**l**) of one experiment. Data are analysed by one-way ANOVA with linear trend test for each subset of buffy coat-ATOs (**d**), two-way ANOVA with Šídák's multiple comparisons test (**g**,**m**) and two-tailed paired t test (**k**). *n*, biological replicates (**b**,**d**,**g**,**k**,**m**); exact *P* values are provided in the Source data.

Fig. 6 | Selective targeting of TNFR2 enhances *in vitro* generation of T cell precursors.

a, Absolute counts of CD45$^+$ cells (n=4) harvested from an ATO, that was aggregated with a normalized amount of 7,500 lin$^-$CD34$^+$CD38$^-$ cord blood HSPCs, at day 10 post culture. **b-d**, Flow cytometric identification of CD45$^+$CD7$^+$CD5$^+$ T cell precursors (n=4) that were generated from HSPCs (**b**), of which some remained undifferentiated and expressed CD34 (**c**). Quantification of the impact of tmTNF density on the cell counts of T cell precursors and undifferentiated CD34$^+$ HSPCs compared to the control (**d**). **e,f**, Flow cytometric analysis of HLA-DR expression on CD1a-expressing T-lineage committed precursors (**e**) and quantification of the cellular fractions (n=4) that were positive (**f**). **g,h**, Flow cytometric analysis of ATO cultures (adult buffy coat lin$^-$CD34$^+$; n=7) at day 10 to determine the impact of 10 ng/mL EHD2-scTNF$_{R2}$ (TNFR2 selective agonist) and 1% tmTNF density on the generation of CD45$^+$CD7$^+$CD5$^+$ T cell precursors (**g**). Relative quantification of the frequency (left) and cell counts (right) of T cell precursors (n=7) generated in TNFR2-activated conditions compared to

the control (**h**). Data are presented as mean±s.d. (**a**,**d**,**f**,**h**) and representatives (**b**,**c**,**e**,**g**) of two independent experiments. Data are analysed by one-way ANOVA with linear trend test (**d**: $P<0.0001$ for undifferentiated $CD34^+$ HSPCs), two-tailed Friedman test with Dunnett's post-hoc analysis (**f**) and one sample two-tailed t test (**h**). *n*, biological replicates (**a**,**d**,**f**,**h**); exact *P* values are provided in the Source data.

Fig. 7 | TNF-activated lymphoid progenitors downregulates expression of IFN-related genes and are competent in T-lineage development.

a-c, UMAP visualization of the 26 cellular clusters (labelled 0 to 25) comprising $CD45^+$ differentiating cells derived from ATOs without (control) and with TNF stimulus (sTNF at 0.25 ng/mL or tmTNF density at 1%) after 10 days of culture (**a**). All clusters identified in **a** were annotated based on their expression profiles of cell type-specific marker genes shown in the dot plot (**b**). The frequency of all the annotated populations of hematopoietic cells (top) and the relative distribution of cells derived from different ATO conditions in each of these populations (bottom) (**c**). **d,e**, UMAP visualization of the enrichment of IFN-related gene signature across all the cellular clusters (**d**). Clustered heatmap shows the average expression of IFN-related genes by lymphoid progenitors (cluster 4 and 15) derived from different ATO conditions (**e**). **f,g**, Flow cytometric analysis at day 13 post secondary ATO cultures (*n*=5) to identify $CD4^+CD8b^+$ thymocytes (**f**). Relative quantification of the frequency (left) and cell counts (right) of $CD4^+CD8b^+$ thymocytes (*n*=5) derived from TNF-activated $CD7^+$ progenitors compared to the control (**g**). **h-j**, Flow cytometric analysis at day 25 post secondary ATO cultures (*n*=5) to identify $CD3^+TCR\alpha\beta^+$ and $CD3^+TCR\gamma\delta^+$ T cells (**h**). Relative quantification of the frequency (left) and cell counts (right) of $CD3^+TCR\alpha\beta^+$ (**i**) and $CD3^+TCR\gamma\delta^+$ (**j**)T cells (*n*=5) derived from TNF-activated $CD7^+$ progenitors compared to the control. Data are presented as mean±s.d. (**g**,**i**,**j**) and representatives (**f**,**h**) of two independent experiments. Data

are analysed by one sample two-tailed t test (**i**,**j**). *n*, biological replicates (**g**,**i**,**j**); exact *P* values are provided in the Source data.

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METHODS

Cell lines

OP9 stromal cells that express GFP only (control) and human Notch ligands were generated and cultured as described previously[25,54]. Jurkat and HL-60 cell lines (ATCC) were cultured as described previously[55,56]. OP9-DLL4-7FS stromal cells (Zúñiga-Pflücker lab[57]) that express human IL-7, SCF and FLT3-L were cultured in MEMα medium containing 5% fetal calf serum (FCS), 100 units/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine. MS5[58,59], previously generated MS5-DLL4[71] and MS5-DLL4/tmTNF (generated herein) stromal cells were cultured in MEMα medium containing 10% FCS, 100 units/mL penicillin and 100 µg/mL

888 streptomycin. *IRF8*$^{+/+}$ and *IRF8*$^{-/-}$ human iPSCs (Zenke lab: corresponding to iPS2[29]) are
889 cultured as described previously. All cell lines were periodically checked for mycoplasma
890 contamination. Information for reagents is provided in Supplementary Table 1.

891

892 **Isolation of human hematopoietic and thymic progenitors**

893 Postnatal thymus was obtained from patients undergoing cardiac surgery with informed consent
894 of parents or guardians. Umbilical cord blood and adult buffy coats were also obtained with
895 informed consent of donors. Fetal liver was obtained from legally interrupted pregnancies with
896 informed consent of the parents. All human cells and tissues were used with permission of and
897 according to the guidelines of the Medical Ethical Commission of Ghent University Hospital,
898 Belgium. Mononuclear cells from thymic total cell suspension, cord blood and buffy coats were
899 isolated by Lymphoprep density gradient centrifugation[60]. Subsequently, CD34^{+} cells were
900 enriched by magnetic-activated cell sorting. Further processing of CD34-enriched cells for
901 downstream experiments is described in the relevant methods. Fetal liver cells, after thawing,
902 were labelled directly with fluorochrome-conjugated antibodies for sorting without CD34
903 enrichment (Supplementary Fig. 1). Information for reagents is provided in Supplementary
904 Table 1.

905

906 **Isolation of murine thymocytes**

907 C57BL/6 mice were kept under specific pathogen-free conditions, with free access to food and
908 water at all times, in individually ventilated cages in a controlled day-night cycle. Whole thymi
909 were isolated from 5 weeks old C57BL/6 mice that had been euthanized with approval of and
910 according to the guidelines of the Medical Ethical Commission of Ghent University Hospital
911 on animal welfare, and grinded directly onto a pre-wet cell strainer to generate single cell

suspension. CD8β-expressing thymocytes were labelled and depleted using streptavidin microbeads. CD8β-depleted thymocytes were used for surface and intracellular flow cytometric staining. Information for reagents and antibodies is provided in Supplementary Table 1 and 2 respectively.

Isolation of human immune cells

To isolate thymic cDCs pDCs, two rounds of negative selection were performed on mononuclear cells derived from thymic total cell suspension. Cells were first stained with unconjugated anti-CD3 and anti-CD8a antibodies, and depleted by using Dynabeads Sheep anti-Mouse IgG. Subsequently, cells that express different lineage markers (lin: CD3, CD14, CD19 and CD56) or CD34 were also labeled and depleted by using streptavidin microbeads. Finally, the remaining cells were stained with antibodies against CD45, CD123, CD1c and HLA-DR to allow sorting of cDCs ($lin^-CD34^-CD45^+HLA\text{-}DR^+CD123^-CD1c^+$) and pDCs ($lin^-CD34^-CD45^+HLA\text{-}DR^+CD123^+CD1c^-$) (Supplementary Fig. 2a). To isolate $CD19^+$ B cells and $CD3^+TCR\alpha\beta^+$ T cells, cord blood-derived mononuclear cells were stained with antibodies against CD19, CD3 and TCRαβ (Supplementary Fig. 2b,c). Human FcR blocking reagent was used to minimize non-specific antibody binding. Information for reagents and antibodies is provided in Supplementary Table 1 and 2 respectively.

Visualization of previously published scRNA-seq data

The *ex vivo* human $CD34^+$ thymocyte scRNA-seq dataset was generated previously[4]. To visualize the expression of *IRF8* and *IL3RA*, violin plots of log2-transformed scRNA-seq count data were generated using the ggplot2 library in R. Heatmaps visualizing pseudobulk scRNAseq count data were generated by summing the counts for individual cells within an

annotated population using the sumCountsAcrossCells function from the Scater library[61]. Subsequently, size factors were calculated using the DESeq2 library[62] and biological replicates were averaged. Finally, the data was scaled using the scale_minmax function from the dynutils library and visualized using the pheatmap library where genes were clustered by using the Ward.D2 algorithm. UCell[63] was used to score the IFN-related gene signature. The average expression values for the IFN-related genes were calculated using the AverageExpression function from the Seurat library[64] and visualized in a heatmap that was constructed using the pheatmap library in R.

Surface and intracellular flow cytometric staining

To immunophenotype *ex vivo* human thymic progenitors, CD34-enriched cells were first stained with antibodies against lineage markers (lin: as defined above), CD4, CD34 and CD1a. Subsequently, dead cells were labeled by fixable viability dye eFluor506. Surface-stained cells were then fixed and permeabilized using Foxp3 Transcription Factor Staining Buffer Set in order to allow intracellular staining of IRF8. Antibodies against surface (CD5, CD7, CD44, CD117, CD123, CD127, CD135 and HLA-DR) and intracellular (CD3, GATA3 and PU.1) markers were fit into the existing staining panel, individually or in combination, depending on the fluorochrome compatibility. Human FcR blocking reagent was included in the staining. Tandem signal enhancer was used to brighten up intracellular signals.

To immunophenotype *ex vivo* murine immature thymocyte, CD8β-depleted thymocytes were stained with antibodies against markers of different lineages (lin: CD122, CD19, NK1.1, CD11b, F4/80, TCRγδ, Gr-1, TER119, CD3e, CD8a and CD11c. Antibodies against c-Kit, CD25 and CD44 were included in the surface staining panel to label different subsets of thymic progenitors (ETP: $lin^-CD44^+c\text{-}Kit^{hi}CD25^-$; DN1 25_{lo}: $lin^-CD44^+c\text{-}Kit^{hi}CD25^{lo}$; DN2a: lin^-

960 $CD44^{+}$c-$Kit^{hi}CD25^{hi}$ and DN2b: $lin^{-}CD44^{+}$c-$Kit^{lo}CD25^{hi}$)[80]. Dead cells were labelled as
961 described above. The surface-stained cells were then fixed and permeabilized as described
962 above in order to allow intracellular staining of IRF8 and GATA3. Mouse FcR blocking reagent
963 was used throughout the staining.

964 Other experiments that involved detection of intracellular markers were performed using
965 similar steps. For analyses that involved cell surface staining only, dead cells were labelled by
966 propidium iodide. For analyses of cells harvested from co-culture experiments, both human and
967 mouse FcR blocking reagents (Miltenyi Biotec) were used. Precision count beads were used
968 where applicable to obtain an absolute cellular count. Information for reagents and antibodies
969 is provided in Supplementary Table 1 and 2 respectively.

970

971 **Viral constructs and transduction**

972 LZRS-IRES-EGFP (empty vector), LZRS-GATA3-IRES-EGFP, LZRS-ICN1-IRES-EGFP,
973 pLKO.1-EGFP (empty vector), pLKO.1-GATA3 shRNA-EGFP and LZRS-DLL4-IRES-EGFP
974 were constructed and described previously[20,56,65,66]. To generate LZRS-IRF8-IRES-EGFP, the
975 *IRF8* insert was released from pIRES2-EGFP-IRF8 by BglII-EcoRI digestion and ligated into
976 the empty vector with BamHI site being destroyed[67]. To generate LZRS-IRF8-ERT2-P2A-
977 EGFP, *IRF8* was first PCR-amplified from pIRES2-EGFP-IRF8 with the addition of 5'-BglII
978 and 3'-XhoI sites, and the omission of stop codon. Subsequently, the PCR-amplified IRF8 insert
979 was ligated into BamHI- and XhoI-digested LZRS-ERT2-P2A-EGFP (courtesy of Karin
980 Weening, Ghent University where the IRES-EGFP sequence in the empty vector was replaced
981 by ERT2-P2A-EGFP sequence using the GeneART Strings DNA fragment from Thermo Fisher
982 Scientific)[68]. To generate LZRS-TNF-IRES-BFP, human *TNF* sequence (NM_000594.4: 178-
983 879 bp) was released from the customized gBlock gene fragment from IDT by BamHI-EcoRI

digestion and ligated into LZRS-IRES-BFP. *IRF8* and *TNF* sequences in the newly generated constructs was validated by Sanger sequencing (Eurofins Genomics). Cell culture supernatants containing retro- (LZRS vectors) and lenti- (pLKO.1 vectors) viral particles were generated and used for transduction as described previously[20,56]. CD34-enriched cells were pre-stimulated with SCF (100 ng/mL),TPO (20 ng/mL) and FLT3-L (100 ng/mL) for 2 days prior transduction with the use of RetroNectin reagent. Spinfection was performed at 890 x g for 90 minutes at 32 ˚C. At day 2 post transduction, cells were stained with antibodies against lineage markers (lin: as defined above) and CD34. $lin^-CD34^+GFP^+$ HSPCs were sorted for immediate analysis or for downstream experiments (Supplementary Fig. 3a-c). For experiments that involved IRF8-related constructs, transduced HSPCs were also stained with antibody against CD123 and sorted for $CD123^-lin^-CD34^+GFP^+$ (Supplementary Fig. 3d,e). To generate MS5-DLL4/tmTNF stromal cells, MS5 cells were transduced with *DLL4* (*EGFP*)- and *TNF* (*BFP*)-encoding retroviral particles with the use of RetroNectin reagent and spinfection. At day 2 post transduction, MS5 cells were sorted based on co-expression of GFP and BFP. Expression of DLL4 and tmTNF were validated by flow cytometry. Information for reagents and antibodies is provided in Supplementary Table 1 and 2 respectively.

OP9 and MS5 co-cultures

To study the regulation of *IRF8* expression by Notch signalling, lin^-CD34^+ HSPCs were sorted and co-cultured with OP9 stromal cells that express GFP only (control) or human Notch ligands. The co-culture medium used was described previously and supplemented with SCF, FLT3-L and IL-7 (all 5 ng/mL) to promote T cell development[54]. At day 3, differentiating $CD45^+$ HSPCs were sorted for analysis by quantitative reverse transcription PCR (RT-qPCR).

To determine the developmental potential of CD123-expressing thymic progenitors, thymic CD34-enriched cells were stained with antibodies against the lineage markers (lin: as defined above), CD4, CD34, CD123 and CD1a, and sorted into 4 subsets ($lin^-CD4^-CD34^+$: $CD123^{hi}CD1a^-$, $CD123^{lo}CD1a^-$, $CD123^-CD1a^-$ and $CD123^-CD1a^+$). All subsets were co-cultured with OP9 stromal cells that express GFP only or DLL4. OP9 co-cultures were supplemented with 20 ng/mL SCF, 100 ng/mL FLT3-L and 20 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) to induce dendritic cell development. OP9-DLL4 co-cultures were supplemented with T-stimulating cytokines as described above.

To investigate the impact of constitutive IRF8 expression on T cell development, CD34-enriched cells were transduced with LZRS-IRES-EGFP (control) or LZRS-IRF8-IRES-EGFP. $CD123^-lin^-CD34^+GFP^+$ HSPCs were sorted and co-cultured with OP9-DLL4 stromal cells in T-stimulating culture conditions as described above.

Similarly, to examine the dose-dependent impact of IRF8 on T cell development, HSPCs transduced with the control or LZRS-IRF8-ERT2-P2A-EGFP were sorted for $CD123^-lin^-CD34^+GFP^+$ and co-cultured with OP9-DLL4 stromal cells. Cells were treated with 4-OHT at day 0 and day 3 post co-culture. For 0 nM condition, cells were treated with the compound solvent at a final concentration equivalent to the highest tested dose of 4-OHT (300 nM). Differentiating HSPCs were analysed or sorted for downstream experiments at the indicated time points.

For co-cultures with OP9-DLL4-7FS stromal cells, half of the existing medium was replaced with fresh medium every 3-4 days till analysis.

To determine the impact of TNFR2 expression on the development of myeloid cells, the sorted HSPCs (Supplementary Fig. 4) were co-cultured with MS5 stromal cells as described previously with the supplementation of 20 ng/mL FLT3-L, 20 ng/mL SCF, 20 ng/mL TPO, 10

ng/mL GM-CSF and 10 ng/mL granulocyte colony-stimulating factor (G-CSF)[69]. Information for reagents and antibodies is provided in Supplementary Table 1 and 2 respectively.

RNA extraction and RT-qPCR

Total RNA from sorted cells was extracted using miRNeasy Micro Kit, with removal of contaminating DNA by DNase digestion, and converted into cDNA using iScript cDNA Synthesis Kit. Whenever it was necessary, target-specific pre-amplification of cDNA was performed using SsoAdvanced SYBR Green Supermix. Real-time PCR reactions were performed using LightCycler 480 SYBR Green I Master Mix and were run on a LightCycler 480 system (Roche). Specific amplification of target was confirmed by melting curve analysis. Gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Information for reagents is provided in Supplementary Table 1. Primer sequences are provided in Supplementary Table 3.

***TCRD* genomic rearrangement**

DNA was extracted from the sorted cells and HL-60 cell line using GenElute Mammalian Genomic DNA Purification Kit. DNA from the $CD123^{hi}(IRF8^{hi})CD1a^{-}$ sorted cells was extracted using homemade tail lysis buffer with proteinase K digestion. Purified DNA was concentrated by ethanol precipitation. Dδ2-Dδ3 recombination was detected using the previously described primer and probe sequences[70]. Dδ2-Dδ3 recombination was normalized against the *Albumin* gene[70] and quantified using the $2^{-\Delta\Delta Ct}$ method. qPCR was performed using the PrimeTime Gene Expression Master Mix with 500 nM of each primer and 200 nM of probe. qPCR was run on a LightCycler 480 system (Roche). Information for reagents is provided in Supplementary Table 1. Primer and probe sequences are provided in Supplementary Table 3.

Analysis of bulk RNA-seq data

Total RNA from the sorted co-cultured cells (Supplementary Fig. 5a) was extracted as described above. mRNA libraries were prepared using QuantSeq 3' mRNA-Seq Library Prep Kit FWD and sequenced as single-end 75 bp reads on the NextSeq 500 System (Illumina). The average total reads per sample is 8.8 ± 1.3 million. All the reads were trimmed using Cutadapt to remove the adaptor sequences, and mapped against Homo sapiens GRCh38 reference genome using STAR[71,72]. A table of read counts for quantifiable genes was generated using RSEM[73]. Differential gene expression analysis between groups of samples was performed using edgeR with batch effect correction due to inter-donor variability[74]. Volcano plots were generated in R. Clustered heatmaps were constructed in R, where normalized counts of significantly differentially expressed genes were rescaled as standard deviations from the mean (Z-scores), between -2 and 2, and clustered based on Pearson correlation. 17 genes that were statistically significantly downregulated in the presence of low level of inducible IRF8 activity constituted the IFN-related gene set. Information for reagents is provided in Supplementary Table 1.

Analysis of bulk ATAC-seq data

Genomic DNA from the sorted co-cultured cells (Supplementary Fig. 5b) was tagmented by Tn5 transposase and DNA libraries were made as described previously[75]. Paired-end sequencing was done on the Illumina NextSeq500 sequencer with a 75 bp read output. The quality of the sequencing was verified with FastQC. Reads were trimmed using NGMerge[76]. Alignment was done using Bowtie2 (v2.2.6) with parameter --very-sensitive, using the human hg38 reference genome[77,78]. Duplicate reads were removed using SAMTools. Peak calling was performed with MACS2 (v2.1.2) and the option --no-model. Significant peaks at an adjusted *p*-

value cut-off of 0.05 were combined into one matrix across all samples, with merging of peaks that show at least 50 percent overlap. The function SummarizeOverlaps of the GenomicAlignments package in R was used for read counting using this matrix and the BAM files[79]. DESeq2 was used to detect significantly differential opened chromatin sites between samples, using a design containing replicate and condition[62]. An adjusted *p*-value of 0.05 was used to retain significant hits. Homer findMotifsGenome was used for motif enrichment analysis on significantly different opened chromatin regions[80]. Information for reagents is provided in Supplementary Table 1.

EMO cultures

To induce hematopoietic specification, EMPs derived from iPSCs were aggregated with MS5-DLL4 stromal cells to form EMOs. EMPs were generated and isolated as described previously[30]. EMOs were assembled and cultured as described previously[30]. After two weeks of hematopoietic induction, EMOs were cultured further in T-stimulating conditions[30] or harvested by forceful pipetting for downstream experiments (flow cytometry, limiting dilution or ATO-spiking analyses).

Limiting dilution analysis

10 (48 wells), 25 (48 wells), 50 (24 wells) and 100 (24 wells) of $TNFR2^{+}CD123^{+}$ hematopoietic progenitors derived from *IRF8*$^{+/+}$ EMOs were sorted (Supplementary Fig. 6) by single cell precision mode for direct co-culture with OP9-DLL4-7FS stromal cells. At day 14, the developmental potential of $TNFR2^{+}CD123^{+}$ hematopoietic progenitors was calculated[81] by analysing each well for the development of T, dendritic cell or both lineages. Only wells in which more than 10 $CD45^{+}$ cells were detected by flow cytometry were scored. The plating

efficiency was 33.3% for 10 cells seeded, 81.3% for 25 cells seeded, 79.2% for 50 cells seeded and 100% for 100 cells seeded.

Detection of sTNF

At day 14, medium from EMO cultures were centrifuged at 1,500 x g at 4 °C for 10 minutes. Aliquots of supernatant (conditioned medium) were stored in protein Lobind tubes at -80 °C for single use[82]. Presence of sTNF in the conditioned medium was detected by LEGENDplex assay with the Human Adipokine Panel. All samples and serially diluted controls were run in duplicates. Data was analysed by LEGENDplex software version 8. Information for reagents is provided in Supplementary Table 1.

Culture of EMOs using conditioned medium

Conditioned medium from void (organoids without EMPs), IRF8 WT and IRF8 KO EMOs, at day 9, 11 and 14 post culture, was collected as described above and used to culture *IRF8*$^{-/-}$ EMOs at day 7, 9 and 11, respectively. *IRF8*$^{+/+}$ and *IRF8*$^{-/-}$ EMOs cultured with fresh medium served as positive and negative controls, respectively. Both fresh and conditioned media were supplemented with 50 ng/mL SCF, 5 ng/mL FLT3-L, 5 ng/mL TPO and 10 μM SB-431542 as described previously[30]. At day 14, cells were harvested for flow cytometric analyses. Information for reagents is provided in Supplementary Table 1.

ATO cultures

ATOs were assembled and cultured as described previously, but with the exception that DLL4 instead of DLL1 was used to support T cell development[9]. As indicated in the individual

experiments, ATOs were assembled using MS5-DLL4 or MS5-DLL4/tmTNF stromal cells or in combination at different ratios. Per ATO, up to 7,500 HSPCs were aggregated with a total amount of 150,000 stromal cells. Whenever indicated, ATOs were cultured and refreshed with medium without the supplementation of 5 ng/mL IL-7, with the supplementation of sTNF (0.25, 5 or 100 ng/mL) or with the supplementation of 10 ng/mL EHD2-scTNF$_{R2}$[37]. Whenever indicated, ATOs were spiked with CD123-expressing hematopoietic cells (1,450 iPSC-derived CD123$^+$ cells and 7,500 HSPCs in an ATO). For secondary ATO cultures, CD45$^+$CD7$^+$ differentiating cord blood HSPCs were sorted into 96-well conical bottom plate using single cell mode (3,500 cells per well). Subsequently, 150,000 stromal cells were added per well to assemble an ATO. At indicated time points, cells were harvested from ATO by forceful pipetting for flow cytometry analyses. Information for reagents is provided in Supplementary Table 1.

Analysis of scRNA-seq data

Cord blood HSPCs were differentiating in ATOs in the absence (control) or presence of TNF stimulus (sTNF at 0.25 ng/mL or tmTNF-expressing stromal cells at 1%). At day 10 post culture, CD45$^+$ cells were sorted from all conditions for scRNA-seq where libraries were prepared and sequenced according to the Chromium Single Cell Gene Expression workflow. Using CellRanger 6.0.1, the sequencing data was mapped against the GRCh38 genome. The filtered feature-barcode matrices were loaded into R. Low quality cells were identified as having less than 200 genes, more than 6000 genes (doublets) or more than 5% mitochondrial reads. Low quality genes were identified as being expressed in less than 3 cells. Both low quality cells and genes were removed. Equal number of cells were sub-sampled from all 3 ATO conditions and integrated (total: 38,439 cells) prior clustering using Seurat[83]. The UMAP method was used to visualize the cell clusters[84]. The two smallest clusters (26: 0.22% and 27:

0.17%) were removed from the original identified 28 clusters. Cell cycle status of the remaining 26 clusters (38,290 cells: 0 to 25) were determined using the CellCycleScoring function from the Seurat library[64]. These 26 clusters were manually annotated based on cell type-specific markers genes that are differentially expressed as determined by using FindAllMarkers from Seurat. Dot plot was used to visualize the expression of cell type-specific marker genes. UCell[63] was used to score the IFN-related gene signature. The average expression values for the IFN-related genes were calculated using the AverageExpression function from the Seurat library[64] and visualized in a clustered heatmap that was constructed using the pheatmap library in R.

Flow cytometric analysis

Fully stained samples were measured on a BD LSR II flow cytometer or a BD FACSymphony A3 Cell Analyzer. Both are equipped with violet (405 nm), blue (488 nm), yellow-green (561 nm) and red (640 nm) lasers. Cells were sorted on a BD FACSAria II or BD FACSAria Fusion flow cytometers. UltraComp eBeads were used to prepare single-color compensation controls for all antibodies used, whereas living and dead Jurkat cells were used as a control to compensate for the spillover of propidium iodide or fixable viability dye eFluor506. Flow cytometric data were visualized and analyzed using BD FlowJo v10. Doublets, aggregates and dead cells were excluded from analyses.

Statistics

GraphPad Prism 9 was used for statistical analyses and graphing. Data with replicates of 3 or more were presented as mean±s.d. All measurements were taken from distinct donors except experiments that involved iPSCs. The Gaussian distribution of data residuals was examined visually by a Quantile-Quantile normality plot and with Shapiro-Wilk statistical test.

Depending on the data normality, parametric or non-parametric statistical tests were applied and indicated in the figure legends.

Data availability

The datasets generated and/or analysed in this study are available in the Gene Expression Omnibus with the following accession numbers: *ex vivo* human $CD34^+$ thymocytes for scRNA-seq (GSE144870), 4-OHT-treated OP9-DLL4 co-cultures for ATAC-seq (GSE179534) and RNA-seq (GSE179381), and TNF-activated ATO cultures for scRNA-seq (GSE211400). GRCh38 reference genome is publicly accessible at the NCBI Datasets Genomes.

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