In Vitro Recapitulation of Murine Thymopoiesis from Single Hematopoietic Stem Cells

Authors
Amélie Montel-Hagen, Victoria Sun, David Casero, ..., Stéphanie C. de Barros, Christopher S. Seet, Gay M. Crooks

Correspondence
gcrooks@mednet.ucla.edu

In Brief
Montel-Hagen et al. develop a murine artificial thymic organoid (M-ATO) system to reproduce thymopoiesis in vitro from bone marrow stem and progenitor cells (HSPCs). This method efficiently recapitulates the phenotypic and transcriptional features of normal murine T cell development even when initiated with a single HSC.

Highlights
- M-ATOs mimic the different stages of normal murine thymopoiesis in multiple strains
- M-ATOs support the key transcriptional transitions during T cell development
- M-ATOs generate mature T cells with a diverse TCR repertoire
- M-ATOs generate the complete trajectory of thymopoiesis from a single HSC

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SUMMARY

We report a serum-free, 3D murine artificial thymic organoid (M-ATO) system that mimics normal murine thymopoiesis with the production of all T cell stages, from early thymic progenitors to functional single-positive (CD8$^+$ and CD4$^+$) TCR$^{ab}$ and TCR$^{gd}$ cells. RNA sequencing aligns M-ATO-derived populations with phenotypically identical primary thymocytes. M-ATOs initiated with $\text{Rag}1^{-/-}$ marrow produce the same differentiation block as seen in the endogenous thymus, and Notch signaling patterns in M-ATOs mirror primary thymopoiesis. M-ATOs initiated with defined hematopoietic stem cells (HSCs) and lymphoid progenitors from marrow and thymus generate each of the downstream differentiation stages, allowing the kinetics of T cell differentiation to be tracked. Remarkably, single HSCs deposited into each M-ATO generate the complete trajectory of T cell differentiation, producing diverse TCR repertoires across clones that largely match endogenous thymus. M-ATOs represent a highly reproducible and efficient experimental platform for the interrogation of clonal thymopoiesis from HSCs.

INTRODUCTION

Commitment to the T cell lineage begins in the thymus from stem and progenitor cells that have trafficked from the bone marrow and is induced by complex spatiotemporal interactions between precursor T cells and thymic epithelial cells, thymic mesenchyme, and other hematopoietic cells (Breed et al., 2018; Rothenberg et al., 2008; Takahama, 2006). Among these interactions, Notch signaling from ligands in the thymic stroma (specifically Delta-like ligand 4 [DLL4]) is critical for the exclusive commitment of the progenitor cells to the T cell lineage (Hozumi et al., 2008; Koch et al., 2008; Taghon et al., 2006).

Several in vitro systems have been developed to try to mimic the thymic microenvironment’s ability to induce and maintain T cell commitment and differentiation (Fan et al., 2015; Hare et al., 1999; Schmitt and Zúñiga-Pflücker, 2002). Among them, the OP9-DL1 monolayer co-culture system revolutionized the field, allowing the commitment of hematopoietic stem and progenitor cells (HSPCs) to the T cell lineage in a dish (Schmitt and Zúñiga-Pflücker, 2002). However, monolayer systems have shown limitations: positive selection and thus production of mature TCR$^{ab}$ cells, especially CD4$^+$ T cells, is very limited. In addition, reproducibility is affected by variations in serum-containing medium.

We have recently developed an artificial thymic organoid (ATO) model of in vitro T cell differentiation from human HSPCs (Seet et al., 2017) as well as human pluripotent stem cells (Montel-Hagen et al., 2019). Human ATOs reproducibly generate mature CD8 and CD4 T cells in vitro using three-dimensional (3D) aggregates of HSPCs with a standardized Notch ligand-expressing stromal cell line in serum-free conditions. However, results using identical conditions with murine cells are suboptimal. We now report a modified ATO system that allows the highly efficient differentiation of murine bone marrow HSPCs to mature T cells in vitro. Murine ATOs (M-ATOs) mimicked normal murine thymopoiesis with the production of all immature and mature T cell subsets, including TCR$^{gd}$ cells and conventional TCR$^{ab}$ T cells (both CD8$^+$ and CD4$^+$) and FoxP3$^+$ cells. Mature cells expressed CD62L, responded to TCR activation, and exhibited a broad TCR V$\beta$ repertoire consistent...
Figure 1. Kinetics of Early T Cell Differentiation in Murine ATOs

(A) Schematic of the M-ATO system.

(B) Representative kinetic analysis of T cell differentiation from bone marrow lineage-negative, Sca1⁺, cKit⁺ (LSK) isolated from C57BL/6 wild-type (WT) mice at the indicated time points, gated on CD45⁺ cells to exclude non-hematopoietic cells. Middle and bottom rows: cells are gated on CD11b⁻/CD10, CD11c⁻/CD10, Gr1⁻/CD10, CD19⁻/CD10, B220⁻/CD10, and NK1.1⁻ (Lin neg) and TCRγδ⁻ cells. Bottom row: cells are further gated on double-negative (DN) cells CD8⁻CD4⁻ to analyze DN subsets: DN1 (CD44⁺CD25⁻), DN2 (CD44⁺CD25⁺), DN3 (CD44⁻CD25⁻), and DN4 (CD44⁻CD25⁻) (Figure S1C).

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with positive selection. RNA sequencing of the different M-ATO-derived T cell subsets revealed a transcriptional profile highly similar to that of primary T cell populations from the murine thymus. M-ATOs could recapitulate thymopoiesis from different subsets of BM and thymus progenitor populations. The high efficiency of the M-ATO system allowed the production of millions of T cells with a diverse repertoire from single highly purified HSCs.

Thus, M-ATOs are a technically simple, highly reproducible, and comprehensive platform to study the full range of murine T cell development and maturation from single HSPCs.

**RESULTS**

**M-ATOs Recapitulate the Early Stages of Murine T Cell Development**

To generate a robust and highly reproducible in vitro system that supports the earliest stages of murine T cell commitment as well as positive selection, we modified our previously developed serum-free ATO model (Montel-Hagen et al., 2019; Seet et al., 2017). MS5 stromal cells transduced with murine DLL4 (hereafter referred to as MS5-mDLL4) were aggregated with murine HSCPs (defined as Lin− Sca1+ cKit+; hereafter referred to as LSK) from fresh or frozen C57BL/6 murine young adult (1–4 months) bone marrow and deployed on a cell culture insert at the air-liquid interface (Figure 1A).

In contrast to human ATOs, we identified DMEM-F12 as the optimal basal medium for murine cultures (Figure S1A). As in the human system, we used the B27 supplement as a replacement for fetal calf serum. This serum-free medium (hereafter referred to as D/F12-B27) was supplemented for the entire culture period, with Fms-related tyrosine kinase 3 (Flt3L), interleukin (IL)-7, ascorbic acid and beta mercapto-ethanol (BME) (Figure 1A); for the first week of culture, Stem Cell Factor (SCF) was also added.

M-ATOs induced commitment of HSPCs to the T cell lineage by week 2, while cells expressing markers of non-T cell lineages (CD11b, CD11c, GR1, CD19, B220, and NK1.1; hereafter referred to as Lin) decreased markedly (Figure 1B). M-ATOs rapidly produced γδ T cells, which later decreased in frequency as conventional TCRβ T cells were generated. The immature single-positive CD8+ (ISP8) and CD8+CD4+ double-positive (DP) populations emerged from the CD8− CD4− double-negative (DN) cells between week 2 and week 3 and were maintained long term (Figures 1B and 1C).

Typically, cell numbers generated in M-ATOs peaked at week 5 with an average 12,000-fold increase when initiated with 500 LSK cells in a single ATO. Cell numbers slightly dropped at week 5 but remained constant until the end of the culture at week 8 (Figure 1C). Interestingly, a lower concentration (2%) of B27 supplement compared with that used in the human system (4%) was beneficial for longer term cultures (Figure S1B).

We analyzed subsets of the CD4−CD8− DN population over time using the standard CD44 and CD25 markers (Godfrey et al., 1993) (Figures 1B, 1D, and 1E). Daily analysis during the first 2 weeks of culture demonstrated progression of the cultures from the DN1 through DN4 stages, with DN3 cells predominating after the first week of culture (Figures 1D and 1E). A detailed analysis of the earliest stages using c-kit expression, as described by the Rothenberg group (Yui et al., 2010), further delineated progression through early thymic progenitors (ETPs), DN2a, and DN2b populations (Figure 1E; Figure S1C). DN3a and DN3b subsets could also be discriminated on the basis of either CD28 expression (Teague et al., 2010) or intracellular TCRβ (iTCRβ) (Taghon et al., 2006; Wilson et al., 1999) (Figure S1D).

**T Cell Maturation in M-ATOs**

Analysis of more mature T cells demonstrated the emergence of CD3+ TCRβ+ cells between week 2 and week 3 of culture. This population was maintained throughout culture and contained CD4−CD8− (DP), CD4−CD8+ (CD8SP), and CD8−CD4+ (CD4SP) mature T cells (Figures 2A and 2B). A population of CD3+CD4+CD8− cells consistent with previously described innate T cells (Brandt and Hedrich, 2018; Yamamoto et al., 2019) was also observed (Figure 2A).

Forty percent to 80% of CD8SP T cells and 5%–15% of CD4SP T cells displayed a CD62L+CD44+ phenotype consistent with conventional mature naive T cell phenotype produced in the normal thymus (Bradley et al., 1994; Budd et al., 1987; Ley and Tedder, 1995) (Figure 2C). CD8SP and CD4SP CD62L+ cells exhibited polyfunctional production of interferon (IFN)−γ, tumor necrosis factor alpha (TNF−α), and IL-2 in response to phorbol 12-myristate13-acetate (PMA) and ionomycin (Figure 2D). Both CD8SP and CD4SP proliferated and upregulated the activation marker CD25 in response to anti-CD3/CD28 and IL-2 stimulation (Figure 2E).

Immunofluorescence staining of whole-mounted M-ATOs at week 4 demonstrated abundant CD4+ and CD8+ T-lineage cells interspersed with MS5-mDLL4 stromal cells (Figure 2F). Further analysis demonstrated the presence of DP, CD8SP, and CD4SP cells (Figures S2A and S2B).

M-ATOs do not contain thymic epithelial cells, which are responsible for the selection of CD4SP in the thymus through major histocompatibility complex (MHC) class II expression. Consistent with this, CD4SP were produced at lower frequency compared with the thymus (Figure 2B). We hypothesized that the ability to generate any CD4SP in M-ATOs was made possible by positive selection mediated via MHC class II-expressing hematopoietic cells generated from the HSPCs that initiated the ATOs. Indeed, the analysis of non-T-lineage cells in 5 week M-ATOs revealed that a small percentage of cells belonged to the myeloid lineage and that some expressed MHC class II molecules. As in the human system, M-ATOs produced some...
dendritic cells, but no B cells were detected in M-ATOs after 5 weeks (Figure S2C).

FoxP3 (Forkhead box P3) is a transcription factor specifically expressed in CD4+CD25+ regulatory T cells (Tregs) and required for their development (Fontenot et al., 2005). Six weeks after initiating M-ATOs with LSK cells from FoxP3 reporter mice, a subset of the CD4SP cells co-expressed GFP (FoxP3) and CD25, suggesting the production of M-ATOs of cells with a Treg phenotype (Figure 2G). Of the two phenotypic Treg precursors that have been described, CD25+FoxP3+ (Marshall et al., 2014; Tai et al., 2013) were at very low frequency, whereas a robust population of CD25+FoxP3− (Lio and Hsieh, 2008) was detected (Figure 2G).

T Cell Differentiation and TCR Diversity in M-ATOs Using Different Murine Backgrounds

The studies described above were performed with bone marrow cells harvested from C57BL/6 mice. There are known intrinsic differences between murine strains in terms of blood and T cell development, including CD4SP/CD8SP predominance (Petkova et al., 2008). To verify that the M-ATO model was applicable to other murine models, we compared the use of LSK cells harvested from the bone marrow of the C3H/He (the strain from which the MS5 stromal line was derived) (Itoh et al., 1989), BALB/c, and FVB murine strains. In all backgrounds, M-ATOs efficiently supported T cell differentiation, although with slightly different kinetics (Figure 3A; Figures S3A−S3C). C3H/He and BALB/c mice generated more CD4SP cells than CD8SP, typical of normal thymopoiesis, whereas in M-ATOs from C57BL/6 and BALB/c, CD8SP predominated relative to CD4SP (Figure 3A; Figures S3A−S3C).

Analysis of TCR diversity via Vβ expression revealed that both the CD8SP and CD4SP cells generated in M-ATOs from the four different murine backgrounds exhibited a broad repertoire (Figure 3B; Figure S3D). Vβ5, 1.5.2, Vβ11, and Vβ12 TCR-bearing T lymphocytes are known to be clonally eliminated, either completely or partially, through negative selection in the thymus of C3H/He and BALB/c mice, but not in C57BL/6 or FVB mice (Abe et al., 1991; Bill et al., 1989, 1990; Gao et al., 1989; Hodes and Abe, 2001; Sugihara et al., 1990; Tomonari et al., 1993; Vaccaro and Hodes, 1989; Woodland et al., 1990, 1991). As expected, Vβ5.1.5.2, Vβ11, and Vβ12 expression was almost undetectable in endogenous thymocytes from C3H/He and BALB/c mice. In contrast, M-ATO-derived cells from C3H/He and BALB/c mice showed significantly increased cells expressing these Vβ segments (Figures 3B and 3C). M-ATOs derived from control C57BL/6 or FVB strains in which Vβ5.1,5.2, Vβ11, and Vβ12 TCR segments are not negatively selected in the thymus showed similar expression of these segments in endogenous thymocytes and M-ATO-derived T cells (Figure 3C). These data are consistent with a lack of negative selection during M-ATO-induced T cell differentiation with the potential for survival of more self-reactive T cells in ATOs.

Transcriptional Analysis of T Cell Differentiation in M-ATOs

Principal-component analysis (PCA) using global genome-wide expression profiles for all populations from M-ATOs, thymus (THY hereafter), and the Immgen dataset (Mingueneau et al., 2013) revealed the major transcriptional events known to operate during T cell development and the relatedness between thymic and M-ATO-derived populations (Figure 4A). The first principal component (PC1; 43% of total variance) fully correlated with the developmental progression from ETPs to positively selected cells. Top PC1-ranked genes (Figure 4B, left) included both legacy hematopoietic progenitor genes (Cd34, Bcl11a, Hhex, Mpo, and Spi1, among others), which were repressed after the ETP/DN2 stages, and genes upregulated during T cell lineage commitment and maturation (lkfz3, Id3, Cd2, Cd4, and Cd8 genes, among others). The second principal component (PC2; 26% of total variance) was associated with genes with peak or repressed expression in the transition from DN3 to DP cells (Figure 4A). PC2 segregated immature (ETF) and mature (single-positive) populations from those involved in the transitions around TCR re-arrangements. Top PC2-ranked genes included Ptxcr, which encodes the pre-T cell antigen receptor alpha, along with the recombination genes Rag1/2, among others (Figure 4B, right).

We next performed unsupervised hierarchical model-based clustering of thymic and M-ATO-derived populations. Because of differences in baseline gene expression, we normalized and compared the pattern of gene expression within and between thymus and M-ATO-derived samples in several ways. To capture all the sources of variability contributing to the PCA map above, we first classified the genes by their overall expression profile, clustering of thymic and M-ATO-derived populations. Because of differences in baseline gene expression, we normalized and compared the pattern of gene expression within and between thymus and M-ATO-derived samples in several ways. To capture all the sources of variability contributing to the PCA map above, we first classified the genes by their overall expression profile, clustering of thymic and M-ATO-derived populations.
Figure 3. T Cell Differentiation and Maturation in M-ATOs from Different Murine Genetic Strains

(A) Frequencies of the different T cell populations generated in M-ATOs from LSK from the C57BL/6, C3H/He, and BALB/c backgrounds shown as percentage of total CD45+ TCRγδ+Lin- cells over time. Error bars denote ± SD (C57BL/6, n = 6; C3H/He, n = 3; BALB/c, n = 3 independent experiments).

(B and C) TCR Vβ expression in T cells from thymus and M-ATOs from different murine genetic strains.

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using an unsupervised approach and stringent thresholds. We then identified a set of most variable genes (2,554 mouse genes) using pairwise statistical tests within (e.g., THY-ETPs versus THY-DN2s), and between (THY-ETPs versus ATO-ETPs) each compartment. To better visualize gene expression trends, clusters were reordered to match peaks in gene expression in the cluster with developmental stages (from ETPs to SPs) (Figure 4C). Hierarchical model-based clustering and heatmap visualization revealed that our approach captured a rich mosaic of expression profiles. As with the PCA, THY and M-ATO expression profiles were highly correlated overall and largely mimicked the profiles of reference populations from Immgen.

Model-based clustering was also performed on the combined dataset (THY-ATO) so that both compartments have the same weight on the obtained groups. We identified a total of 29 gene clusters (Table S1), with distinct expression profiles. The average normalized expression profile for all genes in select clusters are displayed, and representative genes in each cluster are listed (Figure 4D). Individual gene expression levels within each cluster are provided in Table S1. Our unsupervised classification recapitulated the expected behavior of genes with known functions in thymic T cell differentiation, both in THY and M-ATO-derived subsets.

Finally, gene set enrichment analyses (GSEAs) for selected transcriptional transitions revealed that the set of most regulated genes between developmentally proximal populations in THY and M-ATO samples were largely similar (Figure S4), both showing significant enrichment scores when compared against independent thymocyte gene signatures.

**Rag1 Deficiency and Notch Signaling Patterns Are Recapitulated in M-ATOs**

As the M-ATO system closely mirrors phenotypic and transcriptional T cell development in the primary thymus, we next assessed two signaling pathways that are critical for thymopoiesis.

First, we used a Rag1 (recombination activating gene 1)-deficient (Rag1<sup>−/−</sup>) mouse to test whether TCR-dependent developmental checkpoints still applied within the M-ATO system. In M-ATOs generated from Rag1<sup>−/−</sup> marrow LSK cells, DP cells were absent, matching the phenotype of the Rag1<sup>−/−</sup> thymus (Figures 5Ai and 5Aii) (Mombaerts et al., 1992). Both the M-ATO and thymus from Rag1<sup>−/−</sup> mice showed normal progression through the DN1/DN2 to the DN3 stage but a block in transition from DN3 to DN4 (Figures 5Bi and 5Bii). A small population of ISP8 cells (ISP8 cells: CD3<sup>+</sup> CD4<sup>−</sup> CD8<sup>−</sup> CD11<sup>−</sup> V<sub>b</sub> TCR<sup>+</sup>) was also detected in both M-ATO and thymus from Rag1<sup>−/−</sup> mice. Although a DP population emerged later in culture (data not shown), CD3 and TCR<sub>b</sub> were never expressed over the course of the culture, again consistent with Rag1<sup>−/−</sup> thymocytes (Figures 5Ci and 5Cii).

To determine whether Notch signaling during T cell development in the M-ATO resembles that of the endogenous thymus, we used LSK cells from a transgenic Notch reporter (TNR) mouse line (Nowotschin et al., 2013). In both endogenous thymocytes and M-ATO cells, Notch reporter activity fell from the DN2b to the DN3a stage and again during the DN3a to DN3b transition (Figures 5D and 5E); the latter marks the stage during which thymocytes undergo beta selection and when RNA expression of Notch targets is markedly decreased (Mingueneau et al., 2013; Taghon et al., 2006; Teague et al., 2010). Thus, the M-ATO system was able to recapitulate changes in Notch reporter activity in highly defined stages of T cell development.

**The Kinetics of T Cell Differentiation Are Captured by Initiation of M-ATOs with Different Progenitor Populations**

We next interrogated if the M-ATO system was able to recapitulate T cell development when seeded with different phenotypic progenitors in the hematopoietic hierarchy. From C57BL/6 bone marrow, the following phenotypes were tested in M-ATOs: HSC (LSK IL7R<sup>+</sup> CD150<sup>−</sup> CD48<sup>+</sup>), MPPs (multi-potent progenitors) (LSK IL7R<sup>+</sup> CD150<sup>−</sup> CD48<sup>+</sup>), LMPPs (lymphoid-primed MPPs) (LSK IL7R<sup>+</sup> Flk2<sup>+</sup>), and CLPs (common lymphoid progenitors) (Lin<sup>−</sup> c-Kit<sup>−</sup> Sca1<sup>−</sup> IL7R<sup>+</sup> Flk2<sup>+</sup>). In addition, the following populations were isolated from thymus and tested in M-ATOs: ETPs (Lin<sup−</sup> c-Kit<sup>hi</sup> CD44<sup>hi</sup> CD25<sup>−</sup>), DN2 (Lin<sup−</sup> CD4<sup−</sup> CD8<sup−</sup> c-Kit<sup>hi</sup> CD44<sup>hi</sup> CD25<sup>−</sup>), and DN3 (Lin<sup−</sup> CD4<sup−</sup> CD8<sup−</sup> c-Kit<sup>−</sup> CD44<sup−</sup> CD25<sup>−</sup>). Analyses at weeks 1, 2, and 6 were performed to assess the kinetics of T cell development (Figures 6A–6D). At week 1, only M-ATOs initiated with the most T-committed population tested (thymic DN3) had reached the ISP8 and DP stages, while the M-ATO-derived cells initiated from all the other more immature subsets remained at the DN stage (Figures 6A and 6D). However, closer analysis of the DN subsets at week 1 showed that only HSC-initiated M-ATOs remained at the DN1 stage; MPP had generated a mix of DN1-3; LMPP, CLP, and ETP had generated a mix of DN2 and DN3; only DN3 subsets remained in the M-ATOs initiated from DN2 and DN3 populations (Figure 6A; Figure 5S). By week 2 of culture, HSC and MPP-seeded M-ATOs were still almost entirely at the DN stage, whereas M-ATOs initiated from all the lymphoid progenitors (LMPP, CLP, and all thymic progenitors) had begun to produce DP cells and CD3<sup+</sup> TCR<sup+</sup> cells (Figures 6B and 6D). Within the DN cells that remained in cultures, a logical further progression through the subsequent stages was seen from each type of initiating cell (Figure 5S). By week 6, all hematopoietic subsets tested in M-ATOs had produced mature T cells (Figures 6C and 6D). A detailed time-course analysis of T cell differentiation from HSCs is shown in Figures 5S and 5D.

Interestingly, M-ATOs initiated with the most immature stem and progenitor cells (specifically HSCs, MPPs, and LMPPs) generated a higher frequency of CD4<sup+</sup>SP cells (Figure 6C; Figure 5E). CDSP4 output was barely detectable when initiating M-ATOs with CLPs and later populations.
The M-ATO System Supports Thymopoiesis from Isolated Single Cells

The efficiency of T cell differentiation in M-ATOs was next tested using limiting numbers of marrow LSK cells to initiate cultures with a fixed number of MS5-mDLL4 per M-ATO. The cell number and phenotype generated from each M-ATO was similar over a range of 4,000 to as few as 5 initiating LSK cells (Figure 7A). The observation that hematopoietic cells undergo a comparable amount of proliferation regardless of the initiating number of cells in the ATO was consistent throughout extensive studies. We therefore speculate that there are constraints related to the physical size of ATOs, possibly due to gas exchange and nutrient availability.
We next tested the feasibility of generating M-ATOs seeded with single fluorescence-activated cell sorting (FACS)-isolated LSK cells. Of 48 M-ATOs plated, 7 produced sufficient cells for analysis by week 3 (Figure 7B). All 7 of the M-ATOs analyzed revealed a phenotype consistent with T cell commitment (negative for other lineage markers and exhibiting the phenotype of DN populations). Clones showed variations in terms of cell number and stage of differentiation, reflecting the heterogeneity of the LSK HSC/progenitor population. As expected, greater cell numbers were observed in those clones that had already progressed to the DP stage at time of analysis (Figure 7B).

The ability of M-ATOs to support clonal T cell development from highly purified HSC was then tested by seeding single cells from the CD150^+CD48^−phenotypic subset of the LSK population into each M-ATO. At week 3, the cloning efficiency was at least 12% (6 clones out of 48 M-ATOs could be analyzed) (Figure 7C); at this early time point, none of the M-ATOs had progressed past the DN stage (Figure 7C; Figure S6A). Interestingly however, the 3 week clones were not fully synchronized, with some still mostly at the DN1 stage and others already exclusively at the DN3 stage (Figure 7C; Figure S6A), suggesting that the cells within the HSC phenotype were at slightly different states of T lymphoid priming when isolated (Rothenberg, 2011). At weeks 6–7, depending on the technical efficiency of the single-cell seeding, 25%–49% of the M-ATOs showed cell growth, and all clones analyzed revealed T cell commitment, most of them containing DP and TCRβ^+CD3^+CD4SP and CD8SP (Figure 7D; Figures S6B–S6C). Cell output in single HSC-seeded M-ATOs ranged from 2 × 10^3 to 10 × 10^6 by weeks 6–7 (Figure 7D; Figure S6C). Intracellular detection of the Treg-associated transcription factor FOXP3 revealed that 4 of 6 analyzed M-ATOs seeded with a single HSC produced CD4^+CD25^+Foxp3^+Treg-like cells (Figure 7E). Analysis of TCR diversity via Vβ expression by flow cytometry on 12 independent M-ATOs, each generated from a single HSC, revealed that both the CD8SP and CD4SP T cells produced in M-ATOs exhibited a broad repertoire, with a similar pattern of Vβ use seen across M-ATO clones (Figure 7F) and in bulk cultures and normal thymus of the same C57BL/6 strain (Figure 3B).

**DISCUSSION**

We have shown here a simple and powerful *in vitro* model of murine T cell differentiation that faithfully recapitulates the exquisitely controlled progression of key phenotypic and transcriptional events that define normal thymopoiesis. The quantitative and temporal consistency of the M-ATO system provides an experimental platform that can be used to test how extrinsic and intrinsic factors affect the process of T cell commitment and maturation. The M-ATO system allows the rigorous interrogation of T cell potential in highly purified HSC and progenitor populations, including LMPP and CLP from the marrow, and multipotent and T cell committed progenitors in the thymus. Impressively, M-ATOs seeded with single HSCs were able to generate a diverse array of mature thymocytes, including TCRβ^+ , polyclonal CD4SP and CD8SP, and FOXP3^+CD4^+CD25^+ cells.

Despite the remarkable fidelity in the transcriptional profiling of populations from normal thymus and M-ATOs, a few subtle differences were noted. TCRβ^+CD3^+ DP cells from M-ATOs partially overlapped transcriptionally with the more immature TCRβ^−CD3^− DP population from endogenous thymus, possibly because of earlier surface expression of TCRβ/CD3 on DP cells in the M-ATO. Also, basal expression of genes known to be transcriptional targets of Notch signaling was higher in M-ATO populations. Nonetheless, the overall transcriptional profile of Notch targets during T cell development was conserved, and the pattern of Notch signaling seen in the ATO using the TNR mouse model closely mirrored that of *in vivo* thymus.

On the basis of the remarkable TCR Vβ repertoire in M-ATOs seeded with bone marrow progenitors; however, T cell production can be maintained in M-ATOs for several weeks. Thymus transplantation experiments have also shown that thymocytes are able to maintain T cell production without the input of bone marrow progenitors for several weeks, a process called thymus autonomy (de Barros et al., 2013; Martins et al., 2012; Peaudefort et al., 2012). However, although thymus autonomy has been associated with the development of T-ALL (Ballesteros-Arias et al., 2019; Martins et al., 2014) in mice, M-ATO-derived thymocytes maintained a highly diverse TCR Vβ repertoire without evidence of clonal outgrowth for at least 10 weeks of analysis.

The analysis of the TCR Vβ repertoire in M-ATO-derived thymocytes showed that some clones naturally eliminated in the
thymus were present in M-ATOs, strongly suggesting that negative selection does not occur in the organoid system. This finding is consistent with the absence of thymic epithelial cells in ATOs. Although rare dendritic cells are detected in M-ATO cultures, their presence does not appear to be sufficient to induce negative selection. The detection of GFP+CD4+CD25+ cells in M-ATOs generated from FOXP3-reporter mice is phenotypic evidence that Tregs are produced in M-ATOs, but definitive conclusions will require functional studies of these cells.

In the absence of thymic epithelium, positive selection of CD8SP cells is presumed to occur through MHC class I ligands ubiquitously presented by the stromal cells and the hematopoietic cells generated in the cultures. Monolayer murine co-cultures have not permitted the production of CD4SP cells, possibly because of a lack of class II expression or inefficiency of TCR-MHC interactions in these systems. The M-ATO system, however, clearly does generate CD4SP cells. Similar to human HSPC-derived ATOs (Seet et al., 2017), M-ATOs contain a population of hematopoietic cells that express MHC class II, though at a lower frequency than in the normal thymic microenvironment. It is interesting to note that M-ATOs seeded with hematopoietic populations with broad lineage (including myeloid) potential (HSC, MPP, and LMPP) readily generated CD4SP cells, whereas those initiated from progenitors with more restricted, lymphoid only potential (CLP and thymic progenitors) did not, supporting the idea that it is the presence of non-lymphoid lineages that provides the required class II presentation. It is also possible that the 3D structure of the ATO model provides more optimal interactions between MHC class II-expressing cells and T cell precursors than do monolayer systems; of note, other 3D systems (fetal thymic organ cultures and reaggregated primary stromal organoids) also allow positive selection and T cell maturation (Chung et al., 2014; Plum et al., 1994; Poznansky et al., 2000; Robinson and Owen, 1977). In addition, 3D structures may allow easier migration of maturing T cells away from Notch ligand-expressing stromal cells, a process that is seen during migration through the normal cortical-medullary thymic architecture. The M-ATO system offers a simple, reproducible, and modular platform to further explore the specific mechanisms of positive selection and the many other fundamental processes that drive T cell development.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY

Figure 6. M-ATOs Recapitulate T Cell Differentiation from Different Hematopoietic Subsets

(A–C) Different subsets in the hematopoietic hierarchy from HSCs to T cell progenitors were isolated from the bone marrow and the thymus of C57BL/6 WT mice and seeded into M-ATOs. From the bone marrow: HSC (hematopoietic stem cell) (Lin–Sca1+c-Kit+CD48−CD150−IL7R+Flk2+); MPP (multi-potent progenitor) (Lin–Sca1+c-Kit+CD48−CD150−IL7R+Flk2+); LMPP (lymphoid-primed multi-potent progenitor) (Lin–Sca1−c-Kit+IL7R+Flk2+); and CLP (common lymphoid progenitor) (Lin–Sca1+c-Kit+dIL7R+Flk2+). From the thymus: ETP (Lin–CD4−CD8−c-Kit+dCD44−CD25−); DN2 (Lin–CD4−CD8−c-Kit+dCD44−CD25−); and DN3 (Lin–CD4−CD8−c-Kit+dCD44−CD25−). Representative phenotypes of M-ATO-derived cells are shown at weeks 1 (A), 2 (B), and 6 (C). Data are representative of three biological replicates.

(D) Frequencies of T cell populations shown as percentage of total CD45+ TCRβ+ Lin− cells initiated from the different hematopoietic subsets in week 1, week 2, and week 6 M-ATOs. Error bars denote ± SD (n = 3 independent experiments).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2020.108320.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.M.-H., V.S., C.S.S., and G.M.C.; Methodology, A.M.-H. and V.S.; Formal Analysis, D.C.; Investigation, A.M.-H., V.S., S.T., A.Z., N.J., S. Li, S. Lopez, Y.Z., B.C., and C.H.; Data Curation, D.C.; Writing – Original Draft, A.M.-H., V.S., and G.M.C.; Writing – Review & Editing, A.M.-H., V.S., C.S.S., S.C.D., and G.M.C.; Supervision, G.M.C.; Funding Acquisition, G.M.C.
Figure 7. The M-ATO System Supports Full T Cell Differentiation from Isolated Single Cells

(A) Cell numbers and phenotype in week 3 M-ATOs initially seeded with different numbers of LSK isolated from C57BL/6 WT mice (5 to 4,000 cells per ATO). Top: total cell number per M-ATO. Bottom: frequency of cell populations as percentage of CD45+ TCRγδ Lin- cells. Error bars denote ± SD (n = 3 independent experiments).

(B) Cell numbers (top) and phenotype (bottom) at week 3 in M-ATOs seeded with a single LSK cell from bone marrow. Seven independent M-ATOs are shown.

(legend continued on next page)
are detectable in five of six independent experiments. (D) Frequencies of the T cell populations are shown as percentage of total CD45+ TCR

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### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Anti-mouse CD3 (Clone 145-2C11) | Biolegend | Cat# 100312, RRID:AB_312677 |
| Anti-mouse CD4 (Clone RM4-5) | Biolegend | Cat# 100550, RRID:AB_2562099 |
| Anti-mouse CD5 (Clone 53-7.3) | Biolegend | Cat# 100627, RRID:AB_2563930 |
| Anti-mouse CD8a (Clone 53-6.7) | Biolegend | Cat# 100708, RRID:AB_312747, Cat# 100704, RRID:AB_312743 |
| Anti-mouse CD11b (Clone M1/70) | Biolegend | Cat# 101228, RRID:AB_893232 |
| Anti-mouse CD11c (Clone N418) | Biolegend | Cat# 117328, RRID:AB_2129641 |
| Anti-mouse CD16/32 (TruStain FcX) (Clone 93) | Biolegend | Cat# 101320, RRID:AB_1574975 |
| Anti-mouse CD19 (Clone 1D3/CD19) | Biolegend | Cat# 152406, RRID:AB_2629815 |
| Anti-mouse CD25 (PC61) | Biolegend | Cat# 102016, RRID:AB_312865 |
| Anti-mouse CD27 (L.G.3A10) | Biolegend | Cat# 124226, RRID:AB_2565792 |
| Anti-mouse CD28 (37.5) | Biolegend | Cat# 102127, RRID:AB_2650628 |
| Anti-mouse CD44 (IM7) | Biolegend | Cat# 103059, RRID:AB_2571953 |
| Anti-mouse CD45 (clone 30-F11) | Biolegend | Cat# 103116, RRID:AB_312981 |
| Anti-mouse CD45R/B220 (Clone RA3-6B2) | Biolegend | Cat# 103236, RRID:AB_893354 |
| Anti-mouse CD62L (MEL-14) | Biolegend | Cat# 104438, RRID:AB_2563058 |
| Anti-mouse Ly-6G/Ly-6C (Gr-1) (Clone RB6-8C5) | Biolegend | Cat# 108428, RRID:AB_893558 |
| Anti-mouse NK1.1 (Clone PK136) | Biolegend | Cat# 108728, RRID:AB_2132705 |
| Anti-mouse interferon γ (Clone XMG1.2) | Biolegend | Cat# 505806, RRID:AB_315400 |
| Anti-mouse IL-2 (Clone JES6-9H4) | Biolegend | Cat# 503826, RRID:AB_2650897 |
| Anti-mouse TCRβ (Clone H57-597) | Biolegend | Cat# 109234, RRID:AB_2562350, Cat# 109208, RRID:AB_313431 |
| Anti-mouse TCRγδ (Clone GL3) | Biolegend | Cat# 118120, RRID:AB_2562566 |
| Anti-mouse TNFα (Clone MP6-XT22) | Biolegend | Cat# 506339, RRID:AB_2563127 |
| Anti-mouse TER-119 (clone Ter119) | Biolegend | Cat# 116228, RRID:AB_893636 |
| Anti-mouse CD150 (Clone TC15-12F12.2) | Biolegend | Cat# 115941, RRID:AB_2669660 |
| Anti-mouse CD117 (c-KIT) (Clone ACK2) | Biolegend | Cat# 135122, RRID:AB_2562042 |
| Anti-mouse Ly-6A/E (Sca-1) (Clone D7) | Biolegend | Cat# 108114, RRID:AB_493596 |
| Anti-mouse CD48 (Clone HM48-1) | Biolegend | Cat# 103432, RRID:AB_2561463 |
| Anti-mouse CD127 (IL-7R) (Clone REA680) | Miltenyi Biotec | Cat# 130-122-938, RRID:AB_2783928 |
| Anti-mouse CD135 (Flk-2) (Clone A2F10) | Biolegend | Cat# 135306, RRID:AB_1877217 |
| Anti-mDLL4 (clone HMD4-1) | Biolegend | Cat# 130813, RRID:AB_2246026 |
| Anti-GFP (clone FM264G) | Biolegend | Cat# 338001, RRID:AB_1279415 |
| AlexaFluor-594-conjugated donkey anti-rat IgG (H+L) | Jackson ImmunoResearch | Cat# 712-585-150, RRID:AB_2340688 |
| AlexaFluor-488-conjugated donkey anti-rat IgG (H+L) | Jackson ImmunoResearch | Cat# 712-545-150, RRID:AB_2340683 |
| Biotin-SP (long spacer) AffiniPure Goat Anti-Armenian Hamster IgG (H+L) | Jackson ImmunoResearch | Cat# 127-065-160, RRID:AB_2338980 |
| AlexaFluor-594-conjugated Streptavidin | Jackson ImmunoResearch | Cat# 016-580-084, RRID:AB_2337250 |

(Continued on next page)
| **REAGENT or RESOURCE** | **SOURCE** | **IDENTIFIER** |
|-------------------------|------------|----------------|
| **Bacterial and Virus Strains** | | |
| pCCL-c-MNDU3-mDLL4 (lentivirus) | This paper | N/A |
| pCCL-c-MNDU3-mDLL4-IRE5-eGFP (lentivirus) | This paper | N/A |
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| rmIL-2 | Peprotech | Cat# 212-12 |
| rmIL7 | Peprotech | Cat# 217-17 |
| rmSCF | Peprotech | Cat# 250-03 |
| rmFlt3L | Peprotech | Cat# 250-31L |
| Beta Mercapto Ethanol (bME) | Sigma-Aldrich | Cat# M7522 |
| L-Acetic Acid 2-phosphate sequinomagnesium salt hydrate | Sigma-Aldrich | Cat# A8960-5G |
| B27 supplement | ThermoFisher Scientific | Cat# 17504-044 |
| DAPI | Life technologies | Cat# D1306 |
| Formaldehyde | Sigma-Aldrich | Cat# F8775 |
| Vectashield Antifade Mounting Medium | Vector Laboratories | Cat# H1000 |
| **Critical Commercial Assays** | | |
| Direct mouse Lin depletion Kit | Miltenyi Biotec | Cat# 130-110-470 |
| CD8 selection kit | Miltenyi Biotec | Cat# 130-104-075 |
| CD4 isolation kit | Miltenyi Biotec | Cat# 130-104-454 |
| CD62L isolation kit | Miltenyi Biotec | Cat# 130-091-758 |
| Anti-mouse TCR Vβ screening panel | BD Bioscience | Cat# 557004 |
| Cell Stimulation Cocktail (plus protein transport inhibitors) | eBioscience | Cat# 00-4975-03 |
| Intracellular Fixation & Permeabilization Buffer Set | eBioscience | Cat# 88-8824-00 |
| True-Nuclear Transcription Factor Buffer Set | Biolegend | Cat# 424401 |
| Cell Trace Violet cell proliferation assay | Invitrogen | Cat# C34557 |
| Dynabeads Mouse T cell activation CD3/CD28 | GIBCO ThermoFisher | Cat# 11456D |
| RNeasy Micro kit | QIAGEN | Cat# 74004 |
| SMARTer Stranded Total RNA-Seq (Pico) Kit | Clonetech | Cat# 635005 |
| **Deposited Data** | | |
| Raw and analyzed data | This paper | GSE146224 |
| **Experimental Models: Cell Lines** | | |
| MS5-mDLL4 | This paper | N/A |
| MS5-mDLL4-eGFP | This paper | N/A |
| **Experimental Models: Organisms** | | |
| Mouse: C57BL/6J | The Jackson Laboratory | Cat# JAX:000664, RRID:IMSR_JAX:000664 |
| Mouse: B6.Cg-Foxp3^{GFP}^{2+EGFPFTChy/J}, B6-Foxp3^{GFP} | The Jackson Laboratory | Cat# JAX:006772, RRID:IMSR_JAX:006772 |
| Mouse: B6.129S7-Rag1^{ImtmHomy/J} | The Jackson Laboratory | Cat# JAX:002216, RRID:IMSR_JAX:002216 |
| Mouse: BALB/cJ | The Jackson Laboratory | Cat# JAX:000651, RRID:IMSR_JAX:000651 |
| Mouse: C3H/HeJ | The Jackson Laboratory | Cat# JAX:000659, RRID:IMSR_JAX:000659 |
| Mouse: FVB/NJ | The Jackson Laboratory | Cat# JAX:001800, RRID:IMSR_JAX:001800 |
| Mouse: Tg(Cp-HIST1H2BB/Venus)47Hadj/J | The Jackson Laboratory | Cat# JAX:020942, RRID:IMSR_JAX:020942 |

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RESOURCE AVAILABILITY

Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead Contact, Gay Crooks (gcrooks@mednet.ucla.edu).

Materials Availability
The MS5-mDLL4 cell lines generated in this study are available to academic investigators under an MTA with UCLA.

Data and Code Availability
The Gene Expression Omnibus (GEO) accession number for the RNA sequencing data reported in this paper is GEO: GSE146224. A detailed description of data analysis and the software used can be found in Method Details.

EXPERIMENTAL MODELS AND SUBJECT DETAILS

Cell lines
To generate MS5-mDLL4, MS5 cells (Itoh et al., 1989) were transduced with a lentiviral vector encoding full-length murine DLL4 with or without enhanced green fluorescent protein (eGFP). The full-length coding sequence of murine DLL4 was synthesized (Integrated DNA Technologies, Skokie, IL) and cloned into the third-generation lentiviral vector pCCL-c-MNDU3 (gift from Dr. Donald Kohn, UCLA) with or without IRES-linked eGFP expression. Packaging and concentration of lentivirus particles was performed as previously described (Seet et al., 2017). The highest 5% DLL4-expressing cells were sorted by FACS using an anti-DLL4 antibody (Biolegend, Cat# 130813) and passaged in Dulbecco’s Modification of Eagle’s Medium (DMEM) (Cellgro, Cat# 10-017-CV) 10% fetal calf serum (FCS) (Gemini, Cat# 900-208). Stable expression was confirmed by flow cytometry for DLL4 expression or GFP expression after several weeks of culture, as well as qRT-PCR and DNA sequencing.

Mice
All animal experiments were conducted under a protocol approved by the UCLA Chancellor’s Animal Research Committee. This study used 1-4 month-old mice of different backgrounds from Jackson Laboratory (Bar Harbor, Maine): C57BL/6 (Cat# JAX:000664) (including Foxp3-GFP (JAX:006772) mice and RAG1−/− mice (JAX:002216)), C3H/He (JAX:000659), BALB/c (JAX:000651) and FVB (including Transgenic Notch Reporter mice (JAX:020942)) mice. Mice from both sexes were randomly allocated to experimental groups.
METHOD DETAILS

Isolation of murine bone marrow HSPCs
Fresh or frozen bone marrow cells were enriched for hematopoietic stem and progenitor cells by negative cell selection of Lin- cells by magnetic cell sorting (MACS) using Murine Lin depletion Kit (Miltenyi, Auburn CA, Cat# 130-110-470). Hematopoietic stem and progenitor cells were isolated by FACS sorting using the phenotypes as follow: (Lin’ stands for: Ter119-, TCRγδ-, B220-, CD19-, CD11c-, CD11b-, Gr1-, NK1.1-, CD5-, CD4-, CD8-, CD3+).

| Name of cell population | Phenotype |
|-------------------------|-----------|
| LSK (Lin’ Sca1+ cKit+)   | Lin’ Sca1+ cKit+ |
| HSC (Hematopoietic Stem Cell) | Lin’ Sca1+ cKit+ CD48+ CD150+ IL7R+ |
| MPP (MultiPotent Progenitor) | Lin- Sca1+ cKit+ CD48+ CD150+ IL7R+ |
| LMPP (Lymphoid-primed MultiPotent Progenitor) | Lin- Sca1+ cKit+ IL7R+ Flk2+ |
| CLP (Common Lymphoid progenitor) | Lin- Sca1hi cKitlo IL7R+ Flk2+ |

Sorted cells were immediately seeded into MS5-mDLL4 M-ATOs, as described below.

Murine artificial thymic organoid (M-ATO) cultures
M-ATOs were generated as previously described (Seet et al., 2017). MS5-mDLL4 cells were harvested by trypsinization and resuspended in serum free M-ATO culture medium ("D/F12-B27") composed of DMEM-F12 (GIBCO, Cat# 11320033) (in some experiments, RPMI 1640 (CellGro, Cat# 10-040-CV) was used as basal media as indicated), 2% B27 supplement (ThermoFisher Scientific, Grand Island, NY, Cat# 17504-044) (in some experiments, 4% B27 was used as indicated), 30 μM L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (Sigma-Aldrich, St. Louis, MO, Cat# A8960-5G) reconstituted in PBS, 1% penicillin/streptomycin (Gemini Bio-Products, West Sacramento, CA, Cat# 400-109), 1% Glutamax (ThermoFisher Scientific, Grand Island, NY, Cat# 35050-061), 5 ng/ml rmFLT3L (Peprotech, Rocky Hill, NJ, Cat# 250-31L), 5 ng/ml rmIL-7 (Peprotech, Cat# 217-17), 10 ng/ml rmSCF (Peprotech, Cat# 250-03) (SCF was added only for the first week of culture) and beta mercaptoethanol (bME) (0.05mM) (Sigma-Aldrich, Cat# M7522). D/F12-B27 was made fresh weekly. 1.5x10⁵ MS5-mDLL4 cells were plated on a 0.4 μm Millicell transwell insert (EMD Millipore, Billerica, MA; Cat. PICM0RG50) placed in a 6-well plate containing 1 mL D/F12-B27 per well. Medium was changed completely every 3-4 days by aspiration from around the cell insert followed by replacement with 1 mL with fresh D/F12-B27/cytokines. M-ATO cells were harvested by adding FACS buffer (PBS/0.5% bovine serum albumin/2mM EDTA) to each well and briefly disaggregating the M-ATO by pipetting with a 1 mL "P1000" pipet, followed by passage through a 50 μm nylon strainer.

For single cell M-ATO cultures, LSK or HSC cells were isolated as single cells in a 96-conical bottom plate in 200ul of D/F12-B27 medium by flow cytometry cell sorting (FACS ARIA). After centrifugation, 1.5x10⁵ MS5-mDLL4 cells were added in each well of the 96 well plate. The plate was then centrifuged at 300 g for 5 min at 4°C. Supernatants were carefully removed, and each cell pellet was resuspended in 5 μl D/F12-B27 and plated on a 0.4 μm Millicell transwell insert as described above.

Isolation of thymocytes and T cells
Thymic and spleen fragments from the mouse thymus were finely dissected in FACS buffer (PBS/0.5% bovine serum albumin/2mM EDTA) and disrupted by pipetting to release thymocytes into suspension, followed by passage through a 70 μm nylon strainer. Cells were then stained for flow cytometry.

M-ATO-derived T cells were harvested by adding FACS buffer (PBS/0.5% bovine serum albumin/2mM EDTA) to each cell insert and briefly disaggregating the M-ATO by pipetting with a 1 mL "P1000" pipet, followed by passage through a 70 μm nylon strainer. Cells were then stained for flow cytometry.

Flow cytometry cell sorting of thymic and M-ATO-derived T cell populations used the following surface phenotypes: (Lin’ stands here for: Ter119’, TCRγδ’, B220’, CD19’, CD11c’, CD11b’, Gr1’, NK1.1’).

| Name of cell population | Phenotype |
|-------------------------|-----------|
| ETP (Early Thymic Progenitor) | Lin’ CD4’ CD8’ c-Ki67’ CD44hi CD25’ |
| DN2                     | Lin’ CD4’ CD8’ c-Ki67’ CD44hi CD25’ |
| DN3                     | Lin’ CD4’ CD8’ CD11c’ CD11b’ Gr1’ NK1.1’ |

(Continued on next page)
**TCR V\(\beta\) expression analysis by flow cytometry**

Total cells isolated from pooled M-ATOs or murine thymi were stained for Lin\(\text{Ter119, TCR}\ V\) and screened with the anti-mouse TCR V\(\beta\) Screening panel (BD, Biosciences, Cat# 557004). Lin\(\text{CD3}'\text{CD8}'\text{CD4}^{-}\) cells and Lin\(\text{CD3}'\text{CD8}'\text{CD4}^{+}\) cells were gated for analysis, and V\(\beta\) family usage was determined by percent FITC\(^{\text{b}}\), representing a different V\(\beta\) antibody per tube, per the manufacturer’s protocol.

### Name of cell population | Phenotype
---|---
 ISPs8 | Lin' CD4' CD8' CD3' 
 DP early | Lin' CD4' CD8' CD3' TCR\(\gamma\)\(\delta\)* 
 DP late | Lin' CD4' CD8' CD3' TCR\(\gamma\)\(\delta\)* 
 CD8SP | Lin' CD4' CD8' CD3' TCR\(\beta\)^{a} CD62L* 
 CD4SP | Lin' CD4' CD8' CD3' TCR\(\beta\)^{a} CD62L* 

**T cell cytokine assays**

Mature CD8SP and mature CD4SP cells from M-ATOs were isolated by magnetic negative selection using the CD8\(^{+}\) T Cell Isolation Kit (Miltenyi Biotech, Cat# 130-104-075) and the CD4\(^{+}\) T Cell Isolation Kit (Miltenyi Biotech, Cat# 130-104-454) respectively and sorted by FACS or magnetic selection (Miltenyi Biotech, Cat# 130-091-7558) to further isolate CD8SP CD62L\(^{-}\) cells and CD4SP CD62L\(^{-}\) cells. Purified T cell populations were plated in 96-well U-bottom plates in 200 \(\mu\)l RPMI 1640 (CellGro, Cat# 10-040-CV) with 5% fetal calf serum (Hyclone, Cat# SH30070.03) and 0.05mM beta mercaptoethanol (bME) (Sigma-Aldrich, Cat# M7522) with 20 ng/ml rmIL-2 (Peprotech, Rocky Hill, NJ, Cat# 212-12), and plated in 200 \(\mu\)l per well of 96-well round-bottom plates. On day 3, cells were washed and stained for CD3, CD4, and CD8 (Biolegend, San Diego, CA) prior to fixation and permeabilization with an intracellular staining buffer kit (Biolegend, San Diego, CA, Cat# 88-8824-00) and intracellular staining with antibodies against IFN\(\gamma\), TNF\(\alpha\), and IL-2 (Biolegend, San Diego, CA).

**Immunofluorescence imaging of M-ATOs**

M-ATOs were fixed in 4% Formaldehyde (Sigma-Aldrich, Cat# F8775) for 30 minutes at room temperature followed by 3x10 min washes in PBST (0.3% Triton X-100) and a 1-hour block in PBST/BSA (2% BSA). M-ATOs were stained with anti-CD3a (clone 53-6.7; Biolegend), anti-mDLL4 (clone HMD4-1; Biolegend), anti-CD4 (clone RM4-5; Biolegend), and anti-GFP (clone FM264G; Biolegend) at a 1:100 dilution, and anti-CD3 (clone 145-2C11; Biolegend) at a 1:50 dilution overnight at 4°C. Secondary antibodies AlexaFluor-594-conjugated anti-rat IgG (H+L) (Jackson ImmunoResearch, Cat# 712-585-150) or AlexaFluor-488-conjugated anti-rat IgG (H+L) (Jackson ImmunoResearch, Cat# 712-485-153) were added at a 1:200 dilution for 2 hours at room temperature. For anti-mDLL4, anti-hamster biotin (Jackson ImmunoResearch, Cat# 127-065-160) was added at a 1:500 dilution for 2 hours at room temperature, and then AlexaFluor-594-conjugated Streptavidin (Jackson ImmunoResearch, Cat# 016-580-084) was added at a 1:800 dilution for 30 minutes at room temperature. Each M-ATO was mounted individually in Vectashield Antifade Mounting Medium (Vector Laboratories, Cat# H1000) on a concavity microscope slide (Fisher Scientific). Immunofluorescence images were acquired on a Zeiss LSM 880 confocal microscope equipped with Airyscan and Zen software (Zeiss).

**Flow cytometry**

All flow cytometry stains were performed in PBS/0.5% BSA/2 mM EDTA for 20 min on ice. TruStain FcX (Biolegend, San Diego, CA, Cat#101320) was added to all samples for 5 min prior to antibody staining. DAPI (Life technologies, Cat# D1306) was added to all samples (except intracellular staining) prior to analysis.

For intracellular expression analysis, cells were stained for surface markers prior to fixation and permeabilization with the True-Nuclear Transcription Factor Buffer Set (Biolegend, San Diego, CA, Cat# 424401) followed by intracellular staining with antibodies.

Analysis was performed on an LSRII Fortessa, and FACS sorting on FACSARIA or FACSARIA-H instruments (BD Biosciences, San Jose, CA) at the UCLA Broad Stem Cell Research Center Flow Cytometry Core.
For all analyses (except intracellular staining) DAPI+ cells were gated out, and single cells were gated based FSC-H versus FSC-W and SSC-H versus SSC-W.

Anti-mouse antibody clones used for surface and intracellular staining were obtained from Biolegend (San Diego, CA) or Miltenyi Biotech.

Flow cytometry antibody clones used for surface and intracellular staining were obtained from Biolegend (San Diego, CA) or Miltenyi Biotech.

### Flow cytometry antibody

| Antibody Description                           | Source          | Identifier                  |
|-----------------------------------------------|-----------------|-----------------------------|
| Anti-mouse CD3 (Clone 145-2C11)               | Biolegend       | Cat# 100312, RRID:AB_312677 |
| Anti-mouse CD4 (Clone RM4-5)                  | Biolegend       | Cat# 100550, RRID:AB_2562099 |
| Anti-mouse CD5 (Clone 53-7.3)                 | Biolegend       | Cat# 100627, RRID:AB_2563930 |
| Anti-mouse CD8a (Clone 53-6.7)                | Biolegend       | Cat# 100708, RRID:AB_312747 |
| Anti-mouse CD11b (Clone M1/70)                | Biolegend       | Cat# 101228, RRID:AB_893232 |
| Anti-mouse CD11c (Clone N418)                 | Biolegend       | Cat# 117328, RRID:AB_2129641 |
| Anti-mouse CD16/32 (TruStain FcX) (Clone 93) | Biolegend       | Cat# 101320, RRID:AB_1574975 |
| Anti-mouse CD19 (Clone 1D3/CD19)              | Biolegend       | Cat# 152406, RRID:AB_2629815 |
| Anti-mouse CD25 (PC61)                        | Biolegend       | Cat# 102016, RRID:AB_312865 |
| Anti-mouse CD27 (LG.3A10)                     | Biolegend       | Cat# 124226, RRID:AB_2565792 |
| Anti-mouse CD28 (37.5)                        | Biolegend       | Cat# 102127, RRID:AB_2650628 |
| Anti-mouse CD44 (IM7)                         | Biolegend       | Cat# 103059, RRID:AB_2571953 |
| Anti-mouse CD45 (clone 30-F11)                | Biolegend       | Cat# 103116, RRID:AB_312981 |
| Anti-mouse CD45R/B220 (Clone RA3-6B2)        | Biolegend       | Cat# 103236, RRID:AB_893354 |
| Anti-mouse CD62L (MEL-14)                     | Biolegend       | Cat# 104438, RRID:AB_2563058 |
| Anti-mouse Ly-6G/Ly-6C (Gr-1) (Clone RB6-8C5)| Biolegend       | Cat# 108428, RRID:AB_893558 |
| Anti-mouse NK1.1 (Clone PK136)                | Biolegend       | Cat# 108728, RRID:AB_2132705 |
| Anti-mouse interferon γ (Clone XMG1.2)        | Biolegend       | Cat# 505806, RRID:AB_315400 |
| Anti-mouse IL-2 (Clone JES6-SH4)              | Biolegend       | Cat# 503826, RRID:AB_2650897 |
| Anti-mouse TCRb (Clone H57-597)               | Biolegend       | Cat# 109234, RRID:AB_2562350 |
| Anti-mouse TCRγδ (Clone GL3)                  | Biolegend       | Cat# 118120, RRID:AB_2562566 |
| Anti-mouse TNFα (Clone MP6-XT22)              | Biolegend       | Cat# 506339, RRID:AB_2563127 |
| Anti-mouse TER-119 (clone Ter119)             | Biolegend       | Cat# 116228, RRID:AB_893636 |
| Anti-mouse CD150 (Clone TC15-12F12.2)        | Biolegend       | Cat# 115941, RRID:AB_2629680 |
| Anti-mouse CD117 (c-KIT) (Clone ACK2)         | Biolegend       | Cat# 135122, RRID:AB_2562042 |
| Anti-mouse Ly-6A/E (Sca-1) (Clone D7)         | Biolegend       | Cat# 108114, RRID:AB_493596 |
| Anti-mouse CD48 (Clone HM48-1)                | Biolegend       | Cat# 103432, RRID:AB_2561463 |
| Anti-mouse CD127 (IL-7R) (Clone REA680)       | Miltenyi Biotech | Cat# 130-122-328, RRID:AB_2783928 |
| Anti-mouse CD135 (Flk-2) (Clone A2F10)        | Biolegend       | Cat# 135306, RRID:AB_1877217 |
| Anti-mDLL4 (clone HMD4-1)                     | Biolegend       | Cat# 130813, RRID:AB_2246026 |

Flow cytometry data were analyzed with FlowJo software (Tree Star Inc.).

**RNA sequencing (RNA-seq) and data analysis**

RNA was extracted from each of the indicated Thymic or M-ATO-derived populations isolated by FACS, as described above, and total RNA isolated using the RNeasy Micro kit (Qiagen). (Lin” stands here for: Ter119-, TCRγδ-, B220-, CD19-, CD11c-, CD11b-, Gr1-, NK1.1-).
1.5 ng of total RNA was input to generate sequencing libraries with SMARTer Stranded Total RNA-Seq (Pico) Kit (Clonetech, Cat. 635005). Paired-end 150 bp sequencing was performed on an Illumina HiSeq 3000. A total of 32 libraries were multiplexed and sequenced in 5 lanes. Raw sequence files are available at NCBI’s Gene Expression Omnibus (GSE146224). An independent set of RNA-Seq libraries for thymic subsets generated by the Immgen consortium (GSE127267) was downloaded from NCBI’s Gene Expression Omnibus (Bioproject ID PRJNA429735) and analyzed in-house (Table S1).

The STAR ultrafast universal RNA-seq aligner v2.7.0d (Dobin et al., 2013) was used to align the reads to a genome index that includes both the genome sequence (GRCm38 mouse primary assembly) and the exon/intron structure of known mouse gene models (Gencode M20 comprehensive genome annotation). Alignment files were used to generate strand-specific, gene-level count summaries with STAR’s built-in gene counter. Data from the Immgen dataset was not strand-specific. Independent filtering was applied as follows: genes with less than half-count per million in all samples, count outliers or low mappability (< 50bp) were filtered out for downstream analysis (Casero et al., 2015; Love et al., 2014). This masked set included a total of 13760 mouse protein coding genes.

Expression estimates provided throughout were computed in units of fragments per kilobase of mappable length and million counts (FPKMs). Count-based normalized and variance-stabilized data were used for all ordination, differential, and clustering analyses, and all figures unless otherwise noted.

Principal component analysis (PCA, Figures 4A and 4B) was performed with the function prcomp in R (https://www.R-project.org/) using standardize data as input. To facilitate the integration of in-house and external datasets, standardization was performed independently prior to PCA.

Differential expression analyses was performed with DESeq2 (Bioconductor, v3.7, RRID:SCR_015687) (Love et al., 2014). We performed pairwise comparisons between and within thymocyte subsets from both thymic and M-ATO. We defined a set of 2554 variable genes for further analyses as: fold-change greater than 4, Benjamini-Hochberg adjusted Wald test p value less than 0.01 in at least one pairwise test, and a minimum expression of 4 FPKMs in at least one sample. This set of most variable genes was then subjected to model-based clustering using MBCluster.Seq (Si et al., 2014) to classify them based on their overall abundance profile across populations (Figures 4C and 4D). We set the starting number of clusters to 100, and then manually merged them to generate a set of 26 non-redundant gene classes (Table S1).

Functional enrichment for genes selected in the tests and clusters above was performed with Metascape (Zhou et al., 2019). Fold changes between two developmentally proximal populations were employed to perform Gene Set Enrichment Analysis (Subramanian et al., 2005) using gene expression signatures from independent studies (Figure S4). We analyzed the following transitions in both M-ATO-derived and thymic populations from this study: the ETP/DN2 transition from our samples was compared against the “ETP < DN2” (higher expression in DN2) and “ETP > DN2” (higher expression in ETP) signatures from the Molecular Signature Database (MSigDB) and similarly for DN2/DN3 (MSigDB: DN2 > DN3, DN2 < DN3). For the ISP/DPeripheral transition we employed a signature

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### Name of cell population | Number of replicates (n) | Phenotype
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DN2 M-ATO | 2 | Lin− CD4− CD8− c-Kithi CD44hi CD25+
DN3 thymus | 2 | Lin− CD4− CD8− CD24+ CD25+
DN3 M-ATO | 2 | Lin− CD4− CD8− CD24+ CD25+
ISP8 thymus | 2 | Lin− CD4+ CD8− CD3+
ISP8 M-ATO | 2 | Lin− CD4+ CD8− CD3+
DP early thymus | 2 | Lin− CD4+ CD8+ CD3− TCRγδ+
DP early M-ATO | 2 | Lin− CD4+ CD8+ CD3− TCRγδ+
DP late thymus | 2 | Lin− CD4+ CD8+ CD3− TCRγδ+
DP late M-ATO | 2 | Lin− CD4+ CD8+ CD3− TCRγδ+
CD8SP thymus | 2 | Lin− CD4+ CD8+ CD3− TCRγδ+ CD62L+
CD8SP M-ATO | 2 | Lin− CD4+ CD8+ CD3− TCRγδ+ CD62L+
CD4SP thymus | 2 | Lin− CD4+ CD8− CD3+ TCRγδ+ CD62L+
CD4SP M-ATO | 2 | Lin− CD4+ CD8− CD3+ TCRγδ+ CD62L+
for the most regulated genes between double negative and double positive cells (MSigDB: DN > DP, DN < DP). For the DPlate/CD4SP and DPlate/CD8SP transitions we retrieved a signature from the Immgen microarray database (Mingueneau et al., 2013) with genes most differential between DP69+ cells (double positive CD69+ cells, early positive selection) and 4SP24- (CD4+ single positive CD24- mature T cells) or 8SP24- (CD8+ single positive CD24- mature T cells) respectively. Finally, the divergence between our CD4SP and CD8SP single positive populations in both M-ATOs and thymus was compared against a signature from the 4SP24- versus 8SP24- mature thymic T cells contrast from Immgen. Each of the previous signatures typically included a list of the 150 to 200 most up- or downregulated genes between any two populations. All plots in Figures 4 and S4 were generated in MATLAB (MATLAB, version release 2017a, The MathWorks, Inc, RRID:SCR_001622).

QUANTIFICATION AND STATISTICAL ANALYSIS

In all figures, n represents independent experiments and data are represented as mean ± standard deviation (SD) or mean ± standard error of the mean (SEM) as indicated. Statistical analysis was performed using GraphPad Prism software and p-values were calculated from the two-tailed unpaired t test or multiple t test. The p-values are directly indicated on the figure, above the corresponding graphs. *p < 0.05; **p < 0.01; and ***p < 0.001 were considered statistically significant.
Supplemental Information

*In Vitro* Recapitulation of Murine Thymopoiesis
from Single Hematopoietic Stem Cells

Amélie Montel-Hagen, Victoria Sun, David Casero, Steven Tsai, Alexandre Zampieri, Nicholas Jackson, Suwen Li, Shawn Lopez, Yuhua Zhu, Brent Chick, Chongbin He, Stéphanie C. de Barros, Christopher S. Seet, and Gay M. Crooks
Figure S1. T cell development in M-ATOs, related to Figure 1:
(A) Comparison of T cell differentiation in M-ATOs using either RPMI 1640 (black) or DMEM-F12 (pink) as basal medium. Fold expansion of cells (left) and frequencies of different T cell populations (right) are shown. Error bars: +/- SD (n=3 independent experiments, significance: unpaired t-test *p<0.05).
(B) Comparison of T cell differentiation in M-ATOs using either 2% (red) or 4% (grey) of B27 supplement. Cell numbers (left) and frequencies of different T cell populations (right) are shown. Error bars: +/- SD (n=3 independent experiments, significance: unpaired t-test ***p<0.001).
(C) Kinetic analysis of early T cell development (ETP, DN2a and DN2b) in M-ATOs during the first 9 days of culture. Gating strategy is shown on the left. Cells are gated on the DN population (top row) and subgated on CD44\textsuperscript{high} and c-kit\textsuperscript{high} cells (bottom row): (ETP) (c-kit\textsuperscript{high}, CD44\textsuperscript{high}CD25\textsuperscript{-}), DN2a (c-kit\textsuperscript{high}, CD44\textsuperscript{high}CD25\textsuperscript{+}), DN2b (c-kit\textsuperscript{high}, CD44\textsuperscript{dim/low}CD25\textsuperscript{+}). Data is representative of 3 independent experiments.
(D) Frequency of DN3a (CD28\textsuperscript{−} or iTCR\textbeta\textsuperscript{−}) and DN3b (CD28\textsuperscript{+} or iTCR\textbeta\textsuperscript{+}) cells within the DN3 population in the thymus and over the course of M-ATO development from LSK isolated from FVB mice. Error bars: +/- SD (n=3 independent experiments).
Figure S2. T cell and non-T cell development in M-ATOs, related to Figure 2:

(A-B) Immunofluorescence analysis in M-ATOs from LSK isolated from C57BL/6 WT mice representative of 3 independent experiments. Nuclei were stained with DAPI (dark blue). Scale bars, 50μm. (A) CD4 (green) and CD3 (red) expression (top); and CD8 (green) and CD3 (red) (bottom) in week 4 M-ATOs. White arrowhead represents CD3^+CD8^- or CD3^+CD4^- cells. (B) MS5-mDll4 GFP^+ stroma (green), CD4 (cyan), CD8 (red) expression in week 6 M-ATOs. White arrowhead represents CD8^-CD4^+ cells; yellow arrowhead represents CD8^+CD4^- cells. (C) Representative flow cytometry analysis of non-T lineage markers in spleen, thymus and week 5 M-ATOs from LSK isolated from C57BL/6 WT mice.
Figure S3. (related to Figure 3)

(A-B) Representative kinetic analysis of T cell development in M-ATOs from LSK isolated from C3H/He mice (A), BALB/c mice (B) at the indicated time points gated on CD45+ TCRγδ− Lin- cells. Bottom row shows CD4 and CD8 expression gated on CD3+ TCRβ+ cells. Data is representative of 3 biological replicates.

(C) Frequencies of the different T cell populations generated in M-ATOs from LSK from the FVB background shown as percent of total CD45+ TCRγδ− Lin- cells over time. Error bars: +/-SD (n=4 independent experiments). (D) TCR diversity in CD3+CD8SP (top) and CD3+CD4SP (bottom) T cells from thymus and week 6 M-ATOs from LSK isolated from FVB mice. Error bar: +/-SD: FVB thymus n=4, FVB M-ATO n=4 independent experiments.
Figure S4. Gene set enrichment analyses (GSEA) of reference T-cell gene signatures in M-ATO-derived and thymic populations, related to Figure 4:

For each two-population analysis, the x axis represents the pre-ranked list of genes based on fold changes between the two populations from either thymic (Thy-fold, black) or M-ATO-derived (ATO-fold, orange) cells. For instance, for the ETP/DN2 transition, genes highly up-regulated in DN2 cells are positioned to the left. Segment plots (bottom) highlight the position of genes from independent reference signatures (e.g. ETP<DN2 signature) in the sorted vector of fold changes from our samples. The vertical axis in line plots (top) represents the cumulative Enrichment Score (ES) from GSEA, and NES is the overall normalized enrichment score (with FDR=false discovery rate).

Details on the external signatures employed for each transition are provided in Methods.
Figure S5. T cell development in M-ATO from different hematopoietic subsets, related to Figure 6:

(A-B) Representative analysis of (A) early T cell development (ETP, DN2a and DN2b) in week 1 M-ATOs and (B) the DN population in week 2 M-ATOs initiated with the different hematopoietic subsets shown above each graph. Cells are gated on the DN population (top row) and subgated on CD44high and c-kithigh cells (bottom row): (ETP) (c-kithigh, CD44highCD25−) (left gate), DN2a (c-kithigh, CD44highCD25−), DN2b (c-kithigh, CD44dimCD25−). Data are representative of 3 biological replicates.

(C-D) Representative kinetic analysis of (C) early T cell development and (D) mature T cell differentiation in M-ATOs from purified HSCs (LSK CD48−CD150+) cells at the indicated time points (i). Line graphs (ii) show summary of (C) DN, DP, ISP8 population frequencies and (D) TCRβ+CD3+, CD8SP, CD4SP population frequencies as percent of total CD45+ TCRγδ− Lin− cells. Error Bars: +/-SD (n=3 independent experiments).

(E) Analysis of the frequencies of CD4SP and CD8SP cells generated from different hematopoietic subsets (as indicated) at week 6. Ratios between frequencies of CD4SP cells versus CD8SP cells are shown for all hematopoietic subsets (left graph). Right graph shows the comparison of absolute CD4SP cell numbers generated from 1000 LMPP or CLP in M-ATOs. Error bars: +/- SEM (n=3 independent experiments, Significance: unpaired t-tests *p<0.05).
Figure S6. T cell development in M-ATO from single HSCs, related to Figure 7:
(A-B) Flow cytometry analysis of week 3 (A) and week 6 (B) M-ATOs, each initiated with a single HSC (LSK CD48 CD150+). Clone numbers are shown above each graph.
(C) Cell numbers (left) and phenotype (right) in week 6-7 M-ATOs initially seeded with a single (LSK CD48 CD150+) HSC cell. 12 independent M-ATOs are shown from an experiment independent of the one shown in Figure 7D.