



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Initial seeding of the embryonic thymus by immune-restricted lympho-myeloid progenitors

Citation for published version:

Luis, TC, Luc, S, Mizukami, T, Boukarabila, H, Thongjuea, S, Woll, PS, Azzoni, E, Giustacchini, A, Lutteropp, M, Bouriez-Jones, T, Vaidya, H, Mead, AJ, Atkinson, D, Böiers, C, Carrelha, J, Macaulay, IC, Patient, R, Geissmann, F, Nerlov, C, Sandberg, R, de Bruijn, MFTR, Blackburn, C, Godin, I & Jacobsen, SEW 2016, 'Initial seeding of the embryonic thymus by immune-restricted lympho-myeloid progenitors', *Nature Immunology*, vol. 17, no. 12, pp. 1424 - 1437. <https://doi.org/10.1038/ni.3576>

Digital Object Identifier (DOI):

[10.1038/ni.3576](https://doi.org/10.1038/ni.3576)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Nature Immunology

Publisher Rights Statement:

This is a pre-copyedited, author-produced version of an article accepted for publication in Nature Immunology following peer review. The version of record Initial seeding of embryonic thymus by immune-restricted lympho-myeloid progenitors Tiago C. Luis, Sidinh Luc, Takuo Mizukami, Hanane Boukarabila, Supat Thongjuea, Petter S. Woll, Emanuele Azzoni, Alice Giustacchini, Michael Lutteropp, Tiphaine Bouriez-Jones, Harsh Vaidya, Adam J. Mead, Deborah Atkinson, Charlotta Böiers, Joana Carrelha, Iain C. Macaulay, Roger Patient, Frederic Geissmann, Claus Nerlov, Rickard Sandberg, Marella F.T.R. de Bruijn, C. Clare Blackburn, Isabelle Godin, Sten Eirik W. Jacobsen

Nat Immunol. Author manuscript; available in PMC 2017 Jun 1.

Published in final edited form as: Nat Immunol. 2016 Dec; 17(12): 1424–1435. Published online 2016 Oct 3. doi: 10.1038/ni.3576 is available online at: <https://doi.org/10.1038/ni.3576>

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



Initial seeding of the embryonic thymus by immunerestricted lympho-myeloid progenitors

Tiago C Luis^{1,15}, Sidinh Luc^{1–3,15,16}, Takuo Mizukami¹, Hanane Boukarabila¹, Supat Thongjuea^{1,3}, Petter S Woll¹, Emanuele Azzoni³, Alice Giustacchini¹, Michael Lutteropp^{1,3}, Tiphaine Bouriez-Jones¹, Harsh Vaidya⁴, Adam J Mead¹, Deborah Atkinson¹, Charlotta Böiers⁵, Joana Carrelha¹, Iain C Macaulay¹, Roger Patient³, Frederic Geissmann^{6,7}, Claus Nerlov³, Rickard Sandberg⁸, Marella F T R de Bruijn³, C Clare Blackburn⁴, Isabelle Godin^{9–11} & Sten Eirik W Jacobsen^{1,3,12–14} The final stages of restriction to the T cell lineage occur in the thymus after the entry of thymus-seeding progenitors (TSPs). The identity and lineage potential of TSPs remains unclear. Because the first embryonic TSPs enter a non-vascularized thymic rudiment, we were able to directly image and establish the functional and molecular properties of embryonic thymopoiesis-initiating progenitors (T-IPs) before their entry into the thymus and activation of Notch signaling. T-IPs did not include multipotent stem cells or molecular evidence of T cell-restricted progenitors. Instead, single-cell molecular and functional analysis demonstrated that most fetal T-IPs expressed genes of and had the potential to develop into lymphoid as well as myeloid components of the immune system. Moreover, studies of embryos deficient in the transcriptional regulator RBPJ demonstrated that canonical Notch signaling was not involved in pre-thymic restriction to the T cell lineage or the migration of T-IPs.

Multiple T lymphocyte subsets that function as critical mediators of the adaptive immune system are derived from multipotent hematopoietic stem cells (HSCs). While commitment to other blood cell lineages occurs in the adult bone marrow (BM), the final steps toward restriction to the T lymphocyte lineage take place in the thymus¹. Postnatal thymopoiesis is maintained through replenishment by thymus-seeding progenitors (TSPs) migrating from the BM to the thymus¹. Establishing the identity and functional and molecular properties of TSPs is critical for understanding which steps in restriction to the T lymphocyte lineage occur in the BM and subsequently in the thymus and for elucidating the molecular cues that guide this critical lineage-restriction process and the pathways that promote the transition of TSPs from the BM to the thymus. Despite extensive investigation, the identity and lineage potential of postnatal BM-derived TSPs has remained an area of dispute. Because they migrate through the circulation to the vascularized thymus, it has not been possible to directly study or image mammalian BM-derived TSPs before entry into the thymus. Instead, efforts aimed at identifying postnatal TSPs have focused on the characterization of early thymic progenitors (ETPs) already residing within the thymus^{1–4} and therefore have allowed only inferences to be made about TSPs^{1,5}. However, the thymic epithelium expresses Dll4, which encodes the Notch ligand ('Delta-like 4') that activates canonical Notch signaling required for restriction to and development of the T cell lineage^{6–11}, and therefore can rapidly induce restriction of multipotent TSPs to the T cell lineage¹⁰. In mice, the thymic rudiment develops around embryonic day 9 (E9)¹² and is first seeded by hematopoietic cells around E11.5 (ref. 13). In contrast to later fetal and postnatal development, during which TSPs reach the thymus through the circulation⁵, at E11.5 the BM is still not established and the thymic rudiment not yet vascularized. Therefore, the first embryonic TSPs are recruited to the thymic rudiment by migration through the surrounding mesenchyme⁵, guided by chemokines produced in the thymic anlage¹⁴. While Notch signaling has also been suggested to promote the migration of TSPs from adult BM to the thymus¹⁵, its role in the initial seeding of the embryonic thymic rudiment has not been investigated. However, embryonic TSPs have been suggested to be activated by Notch and potentially become restricted to the T cell lineage in the fetal liver (FL)¹⁶ before migrating to the embryonic thymus, although such a possibility has not been

confirmed in Notch-deficient embryos. The thymus harbors multiple non-T cell lineages proposed to have a role in supporting T cell development¹⁷, such as the removal of apoptotic thymocytes by macrophages¹⁸. Fate-mapping studies suggest that in the adult thymus, macrophages develop mostly independently of ETPs^{2,19,20}, whereas their origin in the embryonic thymus²¹ remains to be investigated. While early studies suggested that multipotent HSCs might be responsible for the initial seeding of the embryonic thymus²², subsequent studies failed to support this proposal²³. Other studies have suggested that embryonic TSPs might lack the potential to develop into myeloid cells and B lymphocytes^{16,23,24} and that embryonic TSPs, unlike adult TSPs^{1,3}, might be largely T cell restricted before seeding the thymus. However, most of those studies investigated the thymus at E12 or later, when Notch ligands are expressed and thus might already have restricted the lineage potential of the TSPs¹⁶. However, no studies thus far have characterized the first mammalian T-IPs molecularly and functionally at the single-cell level, before they seed the thymic rudiment and before Notch activation. Here we found that the first T-IP population that migrated through the surrounding mesenchyme to seed the embryonic thymus anlage did not include HSCs. While expressing the green fluorescent protein (GFP) driven by the promoter of the gene encoding the recombinase component RAG1 (the Rag1-GFP reporter)^{3,25}, most T-IPs were not yet restricted to the lymphoid lineage, as they also expressed myeloid transcriptional programs and had myeloid-lineage potential. These results established that embryonic thymopoiesis is initiated by progenitor cells restricted to the lympho-myeloid lineage.

RESULTS

Initial seeding of the embryonic thymic rudiment at E11.25 Colonization of the mouse thymic rudiment has been reported to occur around E11–E11.5 (refs. 13,24). However, developmental staging based on timed mating can vary substantially between and within different litters and between strains²⁶. Moreover, the somite pairs are not clearly visible starting at E11. To accurately define the timing of the initial thymic seeding, we established detailed staging of embryos at E11.0–E11.75, in 0.25-day increments, on the basis of the number of tail somites, using the cloaca as a landmark (Fig. 1a and Supplementary Fig. 1a,b). In the mouse embryo at E11.5, the common thymus and parathyroid rudiments were located bilaterally in the third pharyngeal arches, attached to the endoderm and surface ectoderm²⁷, and adjacent to aortic arches marked by the endothelial marker VE-cadherin and enhanced GFP expressed under control of the promoter for the endothelial gene encoding von Willebrand factor (Vwf-eGFP) (Supplementary Fig. 1c-e). Following that staging by tail somites, we dissected thymic lobes cleanly from surrounding blood vessels to avoid contamination by blood (Fig. 1b and Supplementary Figs. 1e and 2a). VE-Cad⁺ endothelial cells were completely removed by dissection in most explants (Fig. 1b, top) but not all (Fig. 1b, bottom). We dissociated thymic lobes and cultured them on OP9 mouse stromal cells expressing the Notch ligand DL1 (OP9-DL1 cells) to determine the initial colonization of the thymic rudiment by the first hematopoietic cells with the potential to develop into T lymphocytes. This was consistently observed at the stage at which the embryos had 10–12 tail somites (TS10–TS12), corresponding to E11.25–E11.5 (Fig. 1a), when approximately 50% of thymic rudiments contained progenitors with the potential to develop into T cells (Fig. 1c,d). Because activation of Notch might restrict the lineage potentials of T-IPs, we assessed the expression of Dll4 mRNA in PLET1⁺ thymic epithelial cells at E11.5–E12.5 (ref. 12). Consistent with published studies¹⁶, we found that Dll4 was expressed in thymic epithelial cells at E12.5 but had much lower expression at E11.5 (TS11–TS14) (Fig. 1e). Thus, the embryonic thymus was initially seeded at E11.25, before thymic upregulation of Dll4 mRNA. Multipotent HSCs do not initiate embryonic thymopoiesis At E11.5, T-IPs

migrate through the surrounding mesenchyme⁵ to colonize the thymic rudiment, which allows imaging of candidate T-IPs before they enter the thymus and before activation of Notch. Because all definitive HSCs in the FL express Vwf²⁸, while fetal lympho-myeloid-restricted progenitors do not²⁹, we used mice with transgenic expression of the Vwf-eGFP reporter, which express eGFP in HSCs²⁸, to investigate by immunofluorescence whether fetal HSCs seeded the thymic rudiment at TS10–TS12. Although Vwf-eGFP⁺ cells were detected in TS11 FL, they were not detected in the thymus primordium at the time of seeding (TS10–TS12) and were also not detected before (\leq TS9) or after (\geq TS13) the time of seeding (Fig. 2a,b and Supplementary Fig. 2b), which indicated that Vwf-eGFP⁺ HSCs did not migrate to the thymus primordium. In agreement with that conclusion, thymocytes from embryos at E11.5 and E12.5 did not achieve long-term reconstitution of any blood cell lineages in irradiated mice after competitive or non-competitive transplantation, unlike E12.5 FL cells known to contain HSCs (Fig. 2c–e and Supplementary Fig. 2c–f). These results demonstrated that HSCs did not colonize the thymus primordium at TS10–TS12. Initial seeding of the thymic rudiment by Rag1-GFP⁺ progenitors

Next we used Rag1-GFP knock-in mice, in which GFP expression functions as a reporter for transcriptional expression of Rag1 (ref. 25), which marks lymphoid progenitors but not HSCs, to investigate whether lymphoid-restricted progenitors seeded the thymic rudiment. Immunofluorescence analysis demonstrated that low numbers of Rag1-GFP⁺ cells appeared at TS10–TS12, first in the mesenchyme lining the epithelial thymic rudiment and thereafter inside the thymic primordium (Fig. 3a,b and Supplementary Fig. 2g–i). Embryos with Rag1-GFP⁺ cells inside the thymic rudiment consistently also had Rag1-GFP⁺ cells outside or lining the thymus (Fig. 3a,b and Supplementary Fig. 2g–i). Analysis of Rag1-GFP⁺ cells at E11.5 (TS10–TS14) by flow cytometry revealed uniform expression of the pan-leukocyte marker CD45 and the cell-surface cytokine receptors c-Kit (CD117), Flt3 (CD135), IL-7R α and an absence of the cytokine receptor CD25 (IL-2R α) (Fig. 3c,d), similar to results reported for multipotent ETPs in the postnatal thymus³ and for lymphomyeloid-restricted progenitors in the FL at E10.5–11.5 (ref. 29), which lack megakaryocytic-erythroid (MkE) potential. Rag1-GFP⁺ cells also expressed the chemokine receptor CCR9 (Fig. 3c) that is important for embryonic thymus colonization¹⁴. Almost all c-Kit⁺ cells in the thymic rudiment at E11.5 (TS10–TS14) were Rag1-GFP⁺ (data not shown), which suggested that initial seeding of the thymic rudiment was restricted mainly to CD45⁺Lin[–]c-Kit⁺CD25[–]Flt3⁺CCR9⁺ Rag1-GFP⁺ progenitor cells. To investigate whether the Rag1-GFP⁺ progenitor can progress through the thymocyte-progenitor stages from CD4[–]CD8[–] double-negative stage 1 (DN1) through DN4, we cultured dissociated thymic lobes from Rag1-GFP⁺ embryos at E11.5 on OP9-DL1 stroma; this resulted in the generation of DN1–DN4 and CD4⁺CD8⁺ double-positive T cell progenitors (Fig. 3e). Therefore, Rag1-GFP⁺ progenitors seeded the embryonic thymus and were capable of initiating embryonic thymopoiesis. Combined lympho-myeloid potential of Rag1-GFP⁺ T-IPs

To investigate whether the Rag1-GFP⁺ cells that seeded the thymic rudiment at E11.5 were multipotent or T cell restricted, we first performed single-cell RT-PCR analysis. We assessed the expression of genes characteristically expressed by T cells and myeloid cells, comparing their expression in CD45⁺Rag1-GFP⁺ T-IPs at E11.5 with that in lymphoid-primed multipotent progenitors (LMPPs) from FL at E11.5 (ref. 29), as well as with that in DN2 and DN3 thymocytes at E14.5, which are partially T cell restricted and fully T cell restricted, respectively. The expression profiles of T-IPs and LMPPs at E11.5 were very similar, with almost no detectable expression of T cell–restricted genes but with co-expression of genes characteristically expressed by early lymphoid cells (Flt3 and Il7r) and myeloid cells (Fcgr3, Mpo, Csf1r and Csf3r). In contrast, DN3 thymocytes at E14.5 had high expression of genes characteristically expressed by T cells but not those characteristically expressed by myeloid cells¹ (Fig. 4a and Supplementary Fig. 2j,k). These results were

compatible with the idea that the thymus anlage was seeded mainly by Rag1-GFP⁺ T-IPs with sustained myeloid potential. In agreement, CD45⁺Lin⁻cKit⁺CD25⁻Flt3⁺ ETPs at E12.5, which were more frequent at that time point than in the thymus at E11.5, revealed considerable potential to develop into the T cell and myeloid lineages in in vitro cell-culture experiments (Supplementary Fig. 3a–c). Single-cell cultures of CD45⁺Lin⁻cKit⁺CD25⁻Flt3⁺ T-IPs at E11.5 on OP9-DL1 stroma showed that they had combined potential to develop into the T cell and myeloid cell lineages (Fig. 4b–e and Supplementary Fig. 3d–f). Progenitors with the same Rag1-GFP⁺CD45⁺Lin⁻cKit⁺CD25⁻Flt3⁺ phenotype were also identified in the blood of embryos at E11.5 and had combined potential to develop into the T cell and myeloid cell lineages similar to that of T-IPs (Fig. 4f–i and Supplementary Fig. 3g,h). To investigate whether Rag1-GFP⁺ T-IPs contributed physiologically to myeloid cells in the embryonic thymus, we performed fate mapping of cells in Rag1-CreTg/+R26-stop-eYFPFl/+ mice, in which the progeny of Rag1-expressing cells are labeled with enhanced yellow fluorescent protein (eYFP)^{30,31}. Analysis of the thymus rudiments by flow cytometry at TS4–TS8, before the initiation of thymopoiesis, showed that the CD11b⁺F4/80⁺ monocytes-macrophages detected were not eYFP⁺ (Fig. 4j). In contrast, as much as 50% of CD11b⁺F4/80⁺ monocytes-macrophages in the thymus at E14.5 were eYFP⁺ and thus derived from Rag1-expressing progenitors (Fig. 4k–m and Supplementary Fig. 3i). To ascertain that phagocytosis of eYFP⁺ cells did not account for the eYFP signal in Rag1-CreTg/+R26-stop-eYFPFl/+ monocytic cells, we also analyzed by flow cytometry Rag1-GFP reporter mice at E14.5. In these mice, while ETPs and DN2 and DN3 thymocytes were GFP⁺, CD11b⁺F4/80⁺ monocytic cells were GFP⁻ (Supplementary Fig. 3j). Results obtained by single-cell immunofluorescence analysis of the intracellular localization of eYFP in Rag1-CreTg/+R26-stop-eYFPFl/+ E14.5 thymic monocytic cells further indicated the absence of phagocytosis (Supplementary Fig. 3k,l). In addition, following transplantation of a mixture of Rag1-CreTg/+ and R26-stop-eYFPFl/+ (CD45.2⁺) BM cells into wild-type (CD45.1⁺) recipient mice, no eYFP⁺ monocytemacrophages were detected by flow cytometry in the recipients (Supplementary Fig. 3m,n), which suggested that phagocytosed Rag1-Cre expressing thymocytes were not responsible for mediating the recombination and eYFP expression observed in thymic monocytic cells in Rag1-CreTg/+R26-stop-eYFPFl/+ embryos. These results demonstrated that Rag1-expressing progenitors generated thymic monocytes-macrophages. We assessed the potential to develop into megakaryocytes and erythroid cells in vitro in collagen-based and methylcellulose-based assays of dissociated thymic rudiments from embryos at E11.25–E11.75. Despite the abundant M_kE potential in the FL, only 2 of 18 thymuses produced megakaryocyte colonies, and 0 of 17 thymuses generated erythroid colonies (Fig. 5a and Supplementary Fig. 4a–c). To assess the potential of dissected individual thymic lobes to develop into T cells and B cells before the time of seeding (TS8–TS9) and at the time of seeding (TS10–TS11), we plated the dissociated cells on OP9-DL1 and OP9 stroma. No potential to develop into B cells was identified in TS8–TS9 thymic rudiments (Fig. 5b), whereas at TS10–TS11, the frequency of thymuses with the potential to develop into T cells (70%) or B cells (64%) was similar (Fig. 5b,c and Supplementary Fig. 4d,e), which indicated that at least a fraction of the T-IPs that seeded the thymic rudiment had the potential to develop into the T cell as well as B cell lineages. To assess whether the observed potential to develop into B cells might have been derived from progenitors already restricted to the B cell fate, we performed fate-mapping analysis by flow cytometry of cells from Mb1-CreTg/+R26-stop-eYFPFl/+ mice, in which the earliest stage of B cell-committed progenitors and all cells derived from them are marked with eYFP³². No eYFP expression was present in thymocytes from Mb1-CreTg/+R26-stop-eYFPFl/+ embryos at E11.5 (Fig. 5d) and, similar to wild-type FL LMPPs at E11.5, wild-type CD45⁺Lin⁻cKit⁺CD25⁻Flt3⁺ T-IPs at E11.5 lacked expression of mRNA encoding the immunoglobulin

α -chain MB-1 (CD79A), the B cell-specification factor EBF1 and the transcription factor PAX5 (Fig. 5e), which indicated that the observed potential to develop into B cells was derived from multipotent T-IPs at E11.5 and not from B cell-restricted progenitor cells. However, we did not detect consistent potential to develop into B cells in co-cultures of OP9 cells and wildtype CD45⁺Lin⁻c-Kit⁺CD25⁻Flt3⁺ ETPs at E12.5 that had been purified by flow cytometry (data not shown). However, a small fraction of CD45⁺Lin⁻c-Kit⁺CD25⁻Flt3⁺ ETPs at E12.5, from mice with transgenic expression of the antiapoptotic protein MCL-1, which promotes cell survival³, produced B cells (Fig. 5f); this indicated that a fraction of the T-IPs might also have had potential to develop into B cells. Collectively, these results suggested that the T-IPs responsible for initial seeding of the embryonic thymic rudiment were lympho-myeloid progenitors that lacked MkE potential but had the potential to develop into myeloid cells and T cells and, to some degree, B cells as well. Molecular profiling of E11.5 thymopoiesis-initiating progenitors Gene-set-enrichment analysis (GSEA) with published gene sets^{3,33} of RNA-sequencing data²⁹ of CD45⁺Lin⁻c-Kit⁺CD25⁻Flt3⁺ T-IPs at E11.5 and Lin⁻CD45^{lo}VE-Cad⁺c-Kit⁺ hematopoietic stem and progenitor cells (HSPCs)³⁴ from the aorta-gonad-mesonephros (AGM) region at E11.5 (Supplementary Fig. 4f) indicated highly significant upregulation of genes characteristically expressed by early lymphoid cells and downregulation of genes characteristically expressed by MkE cells and HSCs in T-IPs at E11.5, relative to their expression in HSPCs (Fig. 6a-d). Many genes characteristically expressed by myeloid cells were also upregulated in T-IPs at E11.5, relative to their expression in HSPCs (Fig. 6e). The set of genes significantly upregulated in T-IPs at E11.5 relative to their expression in HSPCs at E11.5 showed notable over-representation of those encoding products involved in immune-system-related processes (Supplementary Tables 1 and 2), such as chemotaxis. To further assess the potential of T-IPs to develop into the myeloid and lymphoid lineages, we performed single-cell RT-PCR analysis of CD45⁺Lin⁻c-Kit⁺CD25⁻Flt3⁺ T-IPs at E11.5. All cells analyzed expressed many genes characteristically expressed by lymphoid cells, and 70% of single T-IPs at E11.5 co-expressed genes characteristically expressed by lymphoid cells and myeloid cells but not genes characteristically expressed by MkE cells (Fig. 6f,g). Moreover, genes whose expression defines the earliest T cell-restricted progenitor cells^{3,35}, including *Ptcra*, *Cd3e* and *Bcl11b*, were not expressed by CD45⁺Lin⁻c-Kit⁺CD25⁻Flt3⁺ T-IPs at E11.5 (Figs. 4a and 6f). These molecular findings supported the proposal that T-IPs at E11.5 had combined lympho-myeloid potential but lacked MkE potential. Ontogeny-related changes in thymus-seeding progenitor pathways Principal-component analysis and hierarchical clustering analysis of RNA-sequencing data demonstrated that thymic T-IPs at E11.5 clustered closer to (and between) FL LMPPs at E11.5 (ref. 29) and thymic ETPs at E12.5 (Supplementary Fig. 4g) than to neonatal ETPs (1 week) or adult ETPs (8 weeks) and were more distant from the more multipotent HSPCs from the AGM at E11.5 as well as DN2 cells at E13.5 (Fig. 7a and Supplementary Fig. 5a,b); this indicated that the T-IPs that seeded the thymic anlage might have been derived from FL progenitor cells restricted to the lympho-myeloid lineage and gave rise to the first intra-thymic ETPs. When comparing T-IPs at E11.5 with neonatal and adult ETPs, and the corresponding AGM at E11.5 and neonatal and adult HSC populations, we found that 97 genes were substantially upregulated in the T-IP-ETP populations relative to their expression in the other populations, regardless of age (Fig. 7b). These included *Ccr7* and *Ccr9*, which encode chemokine receptors involved in the migration of fetal and adult TSPs to the thymus^{14,36} (Fig. 7b,c and Supplementary Table 3). 214 genes were uniquely upregulated in E11.5 T-IPs relative to their expression in all HSCs and neonatal and adult ETPs (Fig. 7b and Supplementary Tables 4-6). Among these, expression of *Ccr2*, *Ccr5* and *Ccr6* was notably higher, whereas *Cxcr4* expression was lower, in T-IPs at E11.5 and in ETPs at E12.5 than in neonatal and adult ETPs (Fig. 7c), which indicated the use of distinct

chemokine receptors by embryonic T-IPs. Flow cytometry confirmed the expression of CCR6, CCR7 and CCR9 in CD45+Lin-c-Kit+CD25-Flt3+ T-IPs at E11.5 (Supplementary Fig. 6a). Furthermore, single-cell RT-PCR analysis showed a high degree of co-expression of many genes encoding chemokine receptors (Supplementary Fig. 6b) and high expression of many genes encoding paired immunoglobulin-like receptors (PIRs) in CD45+Lin-c-Kit+CD25-Flt3+ T-IPs at E11.5 (Supplementary Fig. 6c,d). Expression of PIR-encoding genes was almost undetectable in neonatal and adult ETPs, but they were expressed in ETPs at E12.5 (Supplementary Fig. 6c)³⁷. PIR-encoding genes were not expressed in HSCs at E11.5, but were upregulated in FL LMPPs at E11.5, although at lower levels than in E11.5 T-IPs (Supplementary Fig. 6c). Flow cytometry confirmed a greater abundance of PIRA/B in Lin-c-Kit+CD25-Flt3+ Rag1-GFP+ blood progenitor cells at E11.5 than in FL LMPPs and an even greater abundance of PIRA/B in thymic Lin-c-Kit+CD25-Flt3+ Rag1-GFP+ T-IPs at E11.5 and ETPs at E12.5 (Supplementary Figs. 4g and 6e); this indicated that many PIR-encoding genes were co-expressed in lympho-myeloid T-IPs at E11.5. The combined potential to develop into the T cell and myeloid cell lineages noted in OP9-DL1 co-cultures was similar for PIR- and PIR+ E11.5 CD45+Lin-c-Kit+CD25-Flt3+ blood progenitors (Supplementary Fig. 6f). Thus, T-IPs at E11.5 were closely related to FL LMPPs at E11.5 at the molecular level and expressed multiple distinct chemokine receptors and PIR-encoding genes.

Notch is not required for colonization of the thymus at E11.5 GSEA of RNA-sequencing data from T-IPs at E11.5, ETPs at E12.5, and neonatal and adult ETPs with a published gene set³⁸ showed marked enrichment for the expression of genes encoding products related to the Notch pathway in ETPs at E12.5 relative to their expression in T-IPs at E11.5 and a less-distinct additional upregulation of these genes in ETPs from E12.5 to the neonatal period (Fig. 8a). Several genes that are established targets of Notch, including Hes1, Dtx1 and Nrarp, were expressed in ETPs at E12.5 but had low expression in T-IPs at E11.5. The expression of these genes in T-IPs at E11.5 was also notably lower than that in FL LMPPs and HSPCs at E11.5 (Fig. 8b). Whole-mount imaging analysis of RbpjFl/FIVav-CreTg/+Rag1-GFPTg/+ embryos at TS10-TS12 (in which the gene encoding RBPJ, a transcriptional regulator essential for canonical signaling through all Notch receptors⁹, is deleted from hematopoietic cells⁷) showed that the number of Rag1-GFP+ progenitor cells outside, lining and inside the thymus of these mice was similar to that of RbpjFl/FIVav-Cre/+Rag1-GFPTg/+ (control) embryos at TS10-TS12 (Fig. 8c,d and Supplementary Fig. 7a-g); this demonstrated that the migration of T-IPs and their initial seeding of the thymus at E11.5 occurred independently of canonical Notch signaling. Single-cell RT-PCR analysis confirmed efficient deletion of Rbpj in T-IPs (Fig. 8e and Supplementary Fig. 8a). Because most genes that are targets of Notch might also be regulated through Notch-independent mechanisms, we investigated whether the low expression of such genes in T-IPs at E11.5 was independent of canonical activation of Notch. RT-PCR analysis indicated that expression of these genes in single T-IPs from RBPJ-deficient embryos at E11.5 was similar to that in their wild-type counterparts (Fig. 8e and Supplementary Fig. 8b). Moreover, the transcription of genes encoding products associated with lympho-myeloid-lineage priming and T cell-lineage restriction was similar in T-IPs from RBPJ-deficient embryos and wild-type embryos (Fig. 8e and Supplementary Fig. 8c,d), which suggested no involvement of canonical Notch signaling in the pre-thymic lineage restriction of T-IPs. However, subsequent T cell development was blocked at the CD4-CD8-cKit+CD25- ETP stage or CD4-CD8-CD44+CD25- DN1 stage in RBPJ-deficient embryos, in contrast to the normal T cell development in wild-type embryos at E13.5 and E14.5 (Fig. 8f and Supplementary Fig. 8e,f). Furthermore, RBPJ-deficient thymocytes at E11.5 did not progress beyond the CD4-CD8-CD44+CD25- DN1 stage on OP9- DL1 stroma (Supplementary Fig. 8g).

Together these data indicated that unlike its essential role in intra-thymic T lineage restriction, canonical Notch signaling was not required for the initial migration and seeding of the embryonic thymus at E11.25 by Rag1-GFP⁺ T-IPs or in the pre-thymic lineage-restriction of T-IPs.

DISCUSSION

Here we investigated the identity and lineage potential of TSPs that first seeded the thymus rudiment during embryonic development ('T-IPs'). Imaging and purification of T-IPs at E11.5, before their seeding of the thymic rudiment and before thymic upregulation of Dll4, provided a unique opportunity to visualize and characterize T-IPs, rather than ETPs at E12.5 or later^{16,23,39,40}, when there is high expression of Dll4 in the thymus and ETPs are activated via Notch and therefore possibly already restricted in their lineage potential. Through imaging and functional validation, we established that HSCs were not responsible for seeding of the embryonic thymus rudiment at TS8–TS14, a finding further supported by molecular analysis showing that HSCs lack expression of chemokine receptors required for migration to the embryonic thymus^{14,36}. Instead, we found that the first T-IPs (at TS10–TS12 or E11.25–E11.5) expressed Rag1-GFP and genes characteristically expressed by lymphoid cells, before seeding the embryonic thymus rudiment, and progenitors with the same cell-surface phenotype (and lineage potential) could be identified in the circulation at E11.5. Although our findings established a close phenotypic, molecular and functional relationship between T-IPs at E11.5 and PIRA/B⁺ LMPPs at E11.5 in the FL, we cannot rule out the possibility that T-IPs might also migrate from the yolk sac, in which the first LMPPs appear to originate as early as at E9.5 (ref. 29). Whereas published studies have suggested that embryonic TSPs represent largely T cell–restricted progenitors that lack myeloid potential^{16,23,24,40,41}, our molecular and functional single-cell studies demonstrated that a large fraction of Rag1-GFP⁺ T-IPs at E11.5 expressed combined myeloid cell and T cell lineage programs and potential. While the finding that a fraction of T-IPs had only the potential to develop into T cells in OP9-DL1 *in vitro* culture could be compatible with the idea that a fraction of T-IPs are already T cell restricted before entry into the thymus, this might equally reflect the inability of available assays to 'read out' the short-lived myeloid potential of T-IPs with 100% efficiency. Moreover, high-resolution single-cell molecular analysis showed no expression of early T cell–restricted genes, such as *Ptcr*, *Cd3e* and *Bcl11b*, by T-IPs in the embryonic thymus and thus failed to support the idea that T-IPs include T cell–restricted progenitors. In addition, while expression of genes such as *Gata3* (which encodes the transcription factor GATA-3) and *Tcf7* (which encodes the transcription factor TCF-1) has been used to support the proposal that FL and blood might contain T cell–restricted progenitors⁴¹, their expression is not limited to T cell–restricted progenitors. Therefore, proof that T-IPs include T cell–restricted progenitors will require their further purification and characterization. While expression of PIRs has been suggested to mark a subset of T cell–restricted progenitor cells in FL³⁷, PIR⁺Lin[−] c-Kit⁺CD25[−]Flt3⁺ Rag1-GFP⁺ circulating progenitors at E11.5 had combined potential to develop into T cells and myeloid cells similar to that of Lin[−]c-Kit⁺CD25[−]Flt3⁺Rag1-GFP⁺ T-IPs at E11.5, and single Pir⁺ T-IPs co-expressed lymphoid and myeloid transcriptional programs. T-IPs at E11.5 expressed distinct chemokine-receptor-encoding genes. The higher expression of *Ccr2*, *Ccr5*, *Ccr6* and *Ccr7* in T-IPs at E11.5 than in neonatal and adult ETPs is in agreement with published reports on the expression of these genes in CD45⁺ cells isolated from the perithymic mesenchyme at E12.5 (ref. 42) and indicates different use of chemokine receptors at this developmental stage. Together with the expression of known ligands for CCR7, CCR2 and CCR5 in thymic stromal cells at E14.5 (ref. 43), this differential chemokinereceptor-

expression pattern probably reflects the need for distinct migration properties in the colonization of the non-vascularized embryonic thymus rudiment. For example, in agreement with the greater requirement for CCR7 at pre-thymus rudiment vascularization stages, *Ccr7* deficiency results in greater impairment of colonization of the embryonic thymus at early stages of embryonic development⁴⁴, whereas the involvement of CCR2 and CCR6 in embryonic thymus colonization is yet to be functionally explored. The broad expression of PIR-encoding genes and PIR proteins might explain why most T-IPs at E11.5, like the PIR+ fraction of FL LMPPs at E11.5 (ref. 29), lack the potential to develop into the B cell lineage, in contrast to postnatal ETPs³, which, as shown here, lacked *Pir* expression. Nevertheless, we detected potential to develop into the B cell lineage in whole thymic rudiments at TS10–TS12 (E11.25–E11.5), the same time point at which we detected potential to develop into the T cell lineage. Because there are no B cell–restricted progenitors at this stage of development⁴⁵, as confirmed here by fate-mapping and molecular analysis, this finding supports the proposal that T-IP populations seeding the embryonic thymus at TS10–TS12 include some progenitors with combined potential to develop into T cells, B cells and myeloid cells but no potential to develop into the M_kE lineages. The physiological relevance of the myeloid potential of adult ETPs remains in dispute^{19,20}. Given the rarity of T-IPs and what appears to be a rather restricted myeloid potential, this probably does not represent an important pathway for extra-thymic myelopoiesis. However, because the *Rag1*-Cre fate mapping suggested that a large fraction of monocytes-macrophages in the embryonic thymus were derived from TSPs, similar to granulocytes in the adult thymus¹⁹, TSPs might contribute to the generation of intra-thymic monocytes-macrophages, which are suggested to have important roles in thymic development and homeostasis^{17,18}. It is also possible that the sustained myeloid program in TSPs indicates mainly that the final T cell lineage–restriction step takes place in the thymus through loss of myeloid potential. Published studies based on restricted lineage potential and expression of canonical Notch target genes in candidate TSPs in FL and blood^{16,40} have proposed that activation of Notch might restrict the lineage potential of TSPs before colonization of the thymus. However, our molecular and imaging analysis did not provide support for the proposal of a role for canonical Notch signaling in pre-thymic restriction of T-IPs to the T cell lineage or in their migration to the embryonic thymus. In fact, we found no evidence for canonical activation of Notch when we compared the expression of Notch target genes in RBPJ-deficient T-IPs at E11.5 with that in their wild-type counterparts. In conclusion, through careful staging, imaging and single-cell molecular analysis of the first embryonic progenitors migrating to and seeding the thymic rudiment, we have demonstrated that the initial embryonic thymic seeding is mediated by lympho-myeloid-restricted T-IPs. Moreover, we have provided evidence that canonical Notch signaling is not required for the initiation or maintenance of the prethymic restriction of multipotent progenitors to the T cell lineage.

Methods

Methods and any associated references are available in the online version of the paper. Accession codes. GEO: microarray data, GSE76140. Note: Any Supplementary Information and Source Data files are available in the online version of the paper. Acknowledgments We thank N. Sakaguchi (Kumamoto University) for the *Rag1*-GFP mouse line; M. Reth (Max Planck Institute of Immunobiology) for *Cd79atm1(cre)*Reth mice; S. Srinivas (University of Oxford) for R26-stop-eYFP mice; T. Rabbitts (University of Oxford) for *Rag1*-Cre mice; S. Cory (The Walter & Eliza Hall Institute of Medical Research) for *vavP-Mcl1*–transgenic mice; N. Iscove (Ontario Cancer Institute, University Health Network) for W41 mice; A. Cumano (Institut Pasteur) for OP9 and OP9-DL1 stromal cells; Biomedical Services at

Oxford University for animal support; B. Wu for technical assistance; P. Sopp and S-A. Clark (WIMM FACS facility, supported by the MRC HIU; MRC MHU (MC_UU_12009); NIHR Oxford BRC and the John Fell Fund (131/030 and 101/517), the EPA fund (CF182 and CF170) and by WIMM Strategic Alliance awards G0902418 and MC_UU_12025); C. Lagerholm and D. Waithe (Wolfson Imaging Centre Oxford, supported by the MRC via the WIMM Strategic Alliance (G0902418), the MHU (MC_UU_12009), the HIU (MC_UU_12010), the Wolfson Foundation (18272), MRC/BBSRC/EPSC (MR/K015777X/1), and MIC –Nanoscopy Oxford (NanO): Novel Super-resolution Imaging Applied to Biomedical Sciences, Micron (107457/Z/15Z)) and the WIMM Strategic Alliance (G0902418 and MC_UU_12025)). Supported by the Medical Research Council UK (G0801073 and MC_UU_12009/5 to S.E.W.J.), the Swedish Research Council (S.E.W.J.), the Knut och Alice Wallenberg Foundation (WIRM; S.E.W.J.), the Tobias Foundation (S.E.W.J.), StratRegen, KI (S.E.W.J.), EU-FP7 EuroSyStem Integrated projects (S.E.W.J., C.C.B.), the Swedish Research Council Linnaeus (Hemato-Linné grant to S.E.W.J.), the MRC (Molecular Haematology Unit core award to M.F.T.R.d.B.), EMBO-LTF (T.C.L.), Kay Kendall Leukaemia Fund (T.C.L.) and Leukaemia and Lymphoma Research (C.C.B. and A.J.M.). AUTHOR CONTRIBUTIONS T.C.L., S.L. and S.E.W.J. conceptualized and designed the overall research, analyzed the data and wrote the manuscript, which was subsequently reviewed and approved by all authors; T.C.L. did fate-mapping analyses; S.L. and I.G. established and performed embryonic staging experiments; T.C.L., S.L., H.B., M.F.T.R.d.B. and I.G. performed dissections; T.C.L., T.M., H.B. and E.A. did imaging analyses; T.C.L., S.T., C.B. and I.C.M. analyzed RNA-sequencing data; P.S.W. and A.G. processed RNA samples for RNA sequencing; T.C.L., S.L. and P.S.W. performed in vivo transplantation; T.C.L., S.L., M.L. and T.B.-J. carried out in vitro lineage-potential analyses; T.C.L., H.V., A.J.M. and D.A. did single-cell and quantitative PCR analysis; T.C.L., S.L. and J.C. carried out flow-cytometry experiments; T.C.L., S.L., H.B. and A.J.M. performed morphological analysis; and R.P., F.G., C.N., R.S., M.F.T.R.d.B., C.C.B. and I.G. contributed advice and input on experimental design and data analysis. COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

1. Bhandoola, A., von Boehmer, H., Petrie, H.T. & Zúñiga-Pflücker, J.C. Commitment and developmental potential of extrathymic and intrathymic T cell precursors: plenty to choose from. *Immunity* 26, 678–689 (2007).
2. Bell, J.J. & Bhandoola, A. The earliest thymic progenitors for T cells possess myeloid lineage potential. *Nature* 452, 764–767 (2008).
3. Luc, S. et al. The earliest thymic T cell progenitors sustain B cell and myeloid lineage potential. *Nat. Immunol.* 13, 412–419 (2012).
4. Wada, H. et al. Adult T-cell progenitors retain myeloid potential. *Nature* 452, 768–772 (2008).
5. Jenkinson, E.J., Jenkinson, W.E., Rossi, S.W. & Anderson, G. The thymus and T-cell commitment: the right niche for Notch? *Nat. Rev. Immunol.* 6, 551–555 (2006).
6. Koch, U. et al. Delta-like 4 is the essential, nonredundant ligand for Notch1 during thymic T cell lineage commitment. *J. Exp. Med.* 205, 2515–2523 (2008).
7. Han, H. et al. Inducible gene knockout of transcription factor recombination signal binding protein-J reveals its essential role in T versus B lineage decision. *Int. Immunol.* 14, 637–645 (2002).
8. Radtke, F. et al. Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity* 10, 547–558 (1999).
9. Pear, W.S. & Radtke, F. Notch signaling in lymphopoiesis. *Semin. Immunol.* 15, 69–79 (2003).
10. Ciofani, M. & Zúñiga-Pflücker, J.C. The thymus as an inductive site for T lymphopoiesis. *Annu. Rev. Cell Dev. Biol.* 23, 463–493 (2007).
11. Ferrero, I. et al. DL4-mediated Notch signaling is required for the development of fetal $\alpha\beta$ and $\gamma\delta$ T cells. *Eur. J. Immunol.* 43, 2845–2853 (2013).
12. Blackburn, C.C. & Manley, N.R. Developing a new paradigm for thymus organogenesis. *Nat. Rev. Immunol.* 4, 278–289 (2004).
13. Owen, J.J.

& Ritter, M.A. Tissue interaction in the development of thymus lymphocytes. *J. Exp. Med.* 129, 431–442 (1969). 14. Liu, C. et al. Coordination between CCR7- and CCR9-mediated chemokine signals in prevascular fetal thymus colonization. *Blood* 108, 2531–2539 (2006). 15. Yu, V.W. et al. Specific bone cells produce DLL4 to generate thymus-seeding progenitors from bone marrow. *J. Exp. Med.* 212, 759–774 (2015). 16. Harman, B.C. et al. T/B lineage choice occurs prior to intrathymic Notch signaling. *Blood* 106, 886–892 (2005). 17. Klein, L., Kyewski, B., Allen, P.M. & Hogquist, K.A. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). *Nat. Rev. Immunol.* 14, 377–391 (2014). 18. Tacke, R. et al. The transcription factor NR4A1 is essential for the development of a novel macrophage subset in the thymus. *Sci. Rep.* 5, 10055 (2015). 19. De Obaldia, M.E., Bell, J.J. & Bhandoola, A. Early T-cell progenitors are the major granulocyte precursors in the adult mouse thymus. *Blood* 121, 64–71 (2013). 20. Schlenner, S.M. & Rodewald, H.R. Early T cell development and the pitfalls of potential. *Trends Immunol.* 31, 303–310 (2010). 21. Rae, F. et al. Characterisation and trophic functions of murine embryonic macrophages based upon the use of a Csf1r-EGFP transgene reporter. *Dev. Biol.* 308, 232–246 (2007). 22. Péault, B., Khazaal, I. & Weissman, I.L. In vitro development of B cells and macrophages from early mouse fetal thymocytes. *Eur. J. Immunol.* 24, 781–784 (1994). 23. Kawamoto, H., Ohmura, K. & Katsura, Y. Presence of progenitors restricted to T, B, or myeloid lineage, but absence of multipotent stem cells, in the murine fetal thymus. *J. Immunol.* 161, 3799–3802 (1998). 24. Masuda, K. et al. Thymic anlage is colonized by progenitors restricted to T, NK, and dendritic cell lineages. *J. Immunol.* 174, 2525–2532 (2005). 25. Yokota, T. et al. Tracing the first waves of lymphopoiesis in mice. *Development* 133, 2041–2051 (2006). 26. Downs, K.M. & Davies, T. Staging of gastrulating mouse embryos by morphological landmarks in the dissecting microscope. *Development* 118, 1255–1266 (1993). 27. Gordon, J. & Manley, N.R. Mechanisms of thymus organogenesis and morphogenesis. *Development* 138, 3865–3878 (2011). 28. Sanjuan-Pla, A. et al. Platelet-biased stem cells reside at the apex of the haematopoietic stem-cell hierarchy. *Nature* 502, 232–236 (2013). 29. Böiers, C. et al. Lymphomyeloid contribution of an immune-restricted progenitor emerging prior to definitive hematopoietic stem cells. *Cell Stem Cell* 13, 535–548 (2013). 30. McCormack, M.P., Forster, A., Drynan, L., Pannell, R. & Rabbitts, T.H. The LMO2 T-cell oncogene is activated via chromosomal translocations or retroviral insertion during gene therapy but has no mandatory role in normal T-cell development. *Mol. Cell Biol.* 23, 9003–9013 (2003). 31. Srinivas, S. et al. Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Dev. Biol.* 1, 4 (2001). 32. Hobeika, E. et al. Testing gene function early in the B cell lineage in mb1-cre mice. *Proc. Natl. Acad. Sci. USA* 103, 13789–13794 (2006). 33. Chambers, S.M. et al. Hematopoietic fingerprints: an expression database of stem cells and their progeny. *Cell Stem Cell* 1, 578–591 (2007). 34. Rybtsov, S. et al. Hierarchical organization and early hematopoietic specification of the developing HSC lineage in the AGM region. *J. Exp. Med.* 208, 1305–1315 (2011). 35. Rothenberg, E.V., Moore, J.E. & Yui, M.A. Launching the T-cell-lineage developmental programme. *Nat. Rev. Immunol.* 8, 9–21 (2008). 36. Zlotoff, D.A. et al. CCR7 and CCR9 together recruit hematopoietic progenitors to the adult thymus. *Blood* 115, 1897–1905 (2010). 37. Masuda, K. et al. Prethymic T-cell development defined by the expression of paired immunoglobulin-like receptors. *EMBO J.* 24, 4052–4060 (2005). 38. Mercher, T. et al. Notch signaling specifies megakaryocyte development from hematopoietic stem cells. *Cell Stem Cell* 3, 314–326 (2008). 39. Desanti, G.E. et al. Clonal analysis reveals uniformity in the molecular profile and lineage potential of CCR9+ and CCR9- thymus-settling progenitors. *J. Immunol.* 186, 5227–5235 (2011). 40. Ramond, C. et al. Two waves of distinct hematopoietic progenitor cells colonize the fetal thymus. *Nat. Immunol.* 15, 27–35 (2014). 41. Ikawa, T. et al. Identification of the earliest prethymic T-cell progenitors in

murine fetal blood. *Blood* 103, 530–537 (2004). 42. Jenkinson, W.E. et al. Chemokine receptor expression defines heterogeneity in the earliest thymic migrants. *Eur. J. Immunol.* 37, 2090–2096 (2007). 43. Liu, C. et al. The role of CCL21 in recruitment of T-precursor cells to fetal thymi. *Blood* 105, 31–39 (2005). 44. Liu, C. et al. Coordination between CCR7- and CCR9-mediated chemokine signals in prevascular fetal thymus colonization. *Blood* 108, 2531–2539 (2006). 45. Delassus, S. & Cumano, A. Circulation of hematopoietic progenitors in the mouse embryo. *Immunity* 4, 97–106 (1996).