Lymphotoxin α fine-tunes T cell clonal deletion by regulating thymic entry of antigen-presenting cells

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Medullary thymic epithelial cells (mTEC) purge the T cell repertoire of autoreactive thymocytes. Although dendritic cells (DC) reinforce this process by transporting innocuous peripheral self-antigens, the mechanisms that control their thymic entry remain unclear. Here we show that mTEC-CD4⁺ thymocyte crosstalk regulates the thymus homing of SHPS-1⁺ conventional DCs (cDC), plasmacytoid DCs (pDC) and macrophages. This homing process is controlled by lymphotoxin α (LTα), which negatively regulates CCL2, CCL8 and CCL12 chemokines in mTECs. Consequently, Lta-deficient mice have increased expression of these chemokines that correlates with augmented classical NF-κB subunits and increased thymic recruitment of cDCs, pDCs and macrophages. This enhanced migration depends mainly on the chemokine receptor CCR2, and increases thymic clonal deletion. Altogether, this study identifies a fine-tuning mechanism of T cell repertoire selection and paves the way for therapeutic interventions to treat autoimmune disorders.
Thymic clonal deletion, called negative selection, prevents the generation of autoreactive T cells that could induce autoimmunity. The thymus is subdivided into a medulla surrounded by a cortex, both compartments dedicated to specific selection processes. The medulla has a key function in purging the T cell repertoire of self-reactive specificities, through the large diversity of self-antigens (Ag) expressed by medullary thymic epithelial cells (mTECs). The cortex also promotes the deletion of autoreactive T cells. It has been estimated that ~75% of negatively selected cells are deleted at the double-positive (DP) stage in the cortex, and that ~25% are deleted at the single-positive (SP) stage in the medulla. Dendritic cells (DC) are involved in this process since their constitutive ablation results in impaired clonal deletion and fatal autoimmunity. DCs are involved in the deletion of both DP thymocytes in the cortex and SP thymocytes in the medulla. They constitute a heterogeneous population comprising three distinct subsets: CD11c<sup>+</sup>CD8<sup>-</sup> plasmacytoid DCs (pDC) and two CD11c<sup>+</sup> conventional DC (cDC) subsets—CD11c<sup>-</sup>CD8<sup>+</sup>SHPS-1<sup>-</sup> resident and CD11c<sup>+</sup>CD8<sup>-</sup>SHPS-1<sup>+</sup> migratory cDCs. Intrathymically derived resident cDCs, located in close proximity to mTECs, possess the ability to cross-present self-Ags, expressed by mTECs, to thymocytes. Although mTECs express a large array of self-Ags that critically contributes to negative selection, they cannot cover the whole spectrum of self-Ags expressed in peripheral tissues. Migratory cDCs and pDCs reinforce the deletion of autoreactive thymocytes by continuously migrating from the blood to the thymus, where they display peripheral self-Ags that would be otherwise not presented to thymocytes.

While the migration of cDCs and pDCs in the thymus was described to depend on CCR2 and CCR9, respectively, the implication of the thymic microenvironment and more specifically that of mTEC-thymocyte crosstalk in this process remains unknown. Furthermore, although thymic macrophages constitute another type of Ag-presenting cells (APC) that has been previously described to regulate CCL2 and CCL8, their respective contribution in clonal deletion remains elusive. Furthermore, although thymic macrophages are involved in this process since their constitutive ablation results in impaired clonal deletion and fatal autoimmunity. DCs are involved in the deletion of both DP thymocytes in the cortex and SP thymocytes in the medulla. They constitute a heterogeneous population comprising three distinct subsets: CD11c<sup>+</sup>CD8<sup>-</sup> plasmacytoid DCs (pDC) and two CD11c<sup>+</sup> conventional DC (cDC) subsets—CD11c<sup>-</sup>CD8<sup>+</sup>SHPS-1<sup>-</sup> resident and CD11c<sup>+</sup>CD8<sup>-</sup>SHPS-1<sup>+</sup> migratory cDCs. Intrathymically derived resident cDCs, located in close proximity to mTECs, possess the ability to cross-present self-Ags, expressed by mTECs, to thymocytes. Although mTECs express a large array of self-Ags that critically contributes to negative selection, they cannot cover the whole spectrum of self-Ags expressed in peripheral tissues. Migratory cDCs and pDCs reinforce the deletion of autoreactive thymocytes by continuously migrating from the blood to the thymus, where they display peripheral self-Ags that would be otherwise not presented to thymocytes.

To test whether increased numbers of these three cell types could result from enhanced thymic entry, CD45.1 nucleated blood cells were adoptively transferred intravenously (i.v.) into sublethally irradiated OTII-Rag<sup>-/-</sup> and RipmOVAxOTII-Rag<sup>-/-</sup> recipients (Fig. 1e). These donor cells contained increased numbers of SHPS-1<sup>-</sup> cDCs, pDCs and macrophages in the thymus from OTII:RipmOVA compared with OTII:OTII chimeras (Supplementary Fig. 2a–d). Three weeks later, an ~2-fold increase in SHPS-1<sup>-</sup> cDCs, pDCs and macrophages was observed in the thymus from OTII:RipmOVA compared with OTII:OTII chimeras (Supplementary Fig. 2b–d), confirming that the crosstalk between OVA-expressing stromal cells and CD4<sup>+</sup> thymocytes controls the cellularity of these three cell types. This phenomenon was not due to increased proliferation, since similar frequencies of proliferating Ki-67<sup>+</sup> thymocytes can occur. Enhanced thymic recruitment of peripheral DCs and macrophages was observed in OTII-Rag<sup>-/-</sup> mice as well as in OTII:OTII and OTII:RipmOVA chimeras (Fig. 1d, Supplementary Fig. 2c).

Furthermore, although thymic macrophages constitute another type of Ag-presenting cells (APC) that has been previously described to regulate CCL2 and CCL8, their respective contribution in clonal deletion remains elusive. Additionally, although thymic macrophages are involved in this process since their constitutive ablation results in impaired clonal deletion and fatal autoimmunity. DCs are involved in the deletion of both DP thymocytes in the cortex and SP thymocytes in the medulla. They constitute a heterogeneous population comprising three distinct subsets: CD11c<sup>+</sup>CD8<sup>-</sup> plasmacytoid DCs (pDC) and two CD11c<sup>+</sup> conventional DC (cDC) subsets—CD11c<sup>-</sup>CD8<sup>+</sup>SHPS-1<sup>-</sup> resident and CD11c<sup>+</sup>CD8<sup>-</sup>SHPS-1<sup>+</sup> migratory cDCs. Intrathymically derived resident cDCs, located in close proximity to mTECs, possess the ability to cross-present self-Ags, expressed by mTECs, to thymocytes. Although mTECs express a large array of self-Ags that critically contributes to negative selection, they cannot cover the whole spectrum of self-Ags expressed in peripheral tissues. Migratory cDCs and pDCs reinforce the deletion of autoreactive thymocytes by continuously migrating from the blood to the thymus, where they display peripheral self-Ags that would be otherwise not presented to thymocytes.

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L<sub>T</sub>a limits APC thymic entry through CCR2 ligands in mTECs. We investigated the underlying mechanism(s) by which mTEC-CD4<sup>+</sup> thymocyte crosstalk regulates the thymic recruitment of peripheral DCs and macrophages. Since three TNF members, RANKL, CD40L and L<sub>T</sub>a, are implicated in mTEC-thymocyte crosstalk, we examined whether they could be regulated upon Ag-specific interactions with mTECs. In contrast to Tnf<sub>sf</sub>11 (RANKL) and Cd40lg (CD40L), we found that only L<sub>T</sub>a
Data are representative of three independent experiments (*SEM, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 using unpaired Student’s t-test).

**Fig. 1** Ag-specific interactions between mTECs and CD4+ T cells increase the thymic entry of circulating DCs and macrophages. a-c Flow cytometry profiles, frequencies and numbers of cDCs (CD11cintBST-2hi), pDCs (CD11cintBST-2hi), migratory cDCs (CD11cintSHPS-1hi), migratory pDCs (CD49+SHPS-1hi) and macrophages (F4/80) in the thymus from OTII-Rag2−/− and RipmOVAxOTII-Rag2−/− mice. Data are representative of three independent experiments (n = 3 mice per group and per experiment). d Flow cytometry profiles and frequencies of proliferating Ki-67+ thymic DC subsets and macrophages. Data are representative of two independent experiments (n = 3 mice per group and per experiment). e Experimental setup: nucleated blood cells from CD45.1 WT congenic mice were adoptively transferred into sublethally irradiated CD45.2 OTII-Rag2−/− and RipmOVAxOTII-Rag2−/− recipients. Three days after i.v. adoptive transfer (AT), the thymic entry of DCs and macrophages of CD45.1 donor origin was analysed. SL-TBI: sublethal total body irradiation. f-h Flow cytometry profiles, frequencies and numbers of CD45.1 total donor cells (f) as well as cDCs, pDCs (g) and macrophages (h) of CD45.1 donor origin in the thymus from OTII-Rag2−/− and RipmOVAxOTII-Rag2−/− recipients. Control: non-injected irradiated OTII-Rag2−/− mice. Data are representative of three independent experiments (n = 3–4 mice per group and per experiment). d, h MΦ: macrophage. Error bars show mean ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 using unpaired Student’s t-test.
(LTα) was upregulated in OTII CD4+ thymocytes co-cultured with OVA_{323-333}-loaded mTECs (Fig. 2a), suggesting that LTα could be involved in the thymic entry of peripheral APCs mediated by mTEC-CD4+ thymocyte crosslink. To investigate this hypothesis, we first evaluated on thymic sections the area occupied by CD11c+ cells in the cortex and medulla and found that DC enrichment in the medulla was increased in LTα−/− mice (Supplementary Fig. 6a). Consistently, flow cytometry analyses revealed that LTα−/− mice had increased frequencies and numbers of thymic cDCs and pDCs compared with wild-type (WT) mice (Fig. 2b). Furthermore, although numbers of CD8αhiSHPS-1− resident cDCs were unaltered, numbers of CD8αhiSHPS-1+ migratory cDCs were increased in these mice (Fig. 2c). Frequencies and numbers of F4/80+CD11b+ macrophages were also higher in LTα−/− thymi than in WT thymi (Supplementary Fig. 6b). In line with a previous study, we observed that CD8αhiSHPS-1+ cDCs and pDCs were more proliferative than CD8αhiSHPS-1− cDCs (Supplementary Fig. 6c). Similar frequencies of proliferating Ki-67+ cells were also observed in LTα−/− thymi than in WT thymi (Supplementary Fig. 6d). In line with a previous study, we observed that CD8αhiSHPS-1+ cDCs and pDCs were more proliferative than CD8αhiSHPS-1− cDCs (Supplementary Fig. 6c).
an increased cell proliferation, suggesting a role for LTα in repressing thymus homing of peripheral DCs and macrophages. To demonstrate this hypothesis, sublethally irradiated WT and Ltα−/− recipients were adoptively transferred with CD45.1 nucleated blood cells (Fig. 2e). Three days later, we found increased frequencies and numbers of CD45.1 donor cells in LTα−/− recipients were adoptively transferred with CD45.1 nucleated blood cells (Fig. 2e). Three days later, we found increased frequencies and numbers of CD45.1 donor cells in LTα−/− mice (Fig. 3b). CCL2 is a major chemoattractant that is produced by a variety of cell types and is involved in the trafficking of immune cells. We hypothesised that LTα could regulate the expression of key chemokines involved in the thymus homing of DCs and macrophages. Because CCR9 has been implicated in the thymic entry of peripheral pDCs, we first assessed the expression of its ligand, CCL2, which was unaltered in the total thymus and purified mTECs from LTα−/− mice (Fig. 3a). Since we and others previously showed that CCL2 overexpression in the thymus leads to increased thymic pDC cellularity and that its receptor CCR2 is involved in thymic SHPS-1+ cDC and pDC homeostasis, we hypothesised that LTα could modulate CCL2 expression. Strikingly, CCL2 expression was substantially higher in both total thymus (p < 0.05 by a two-tailed Mann–Whitney test) and purified mTECs (p < 0.05 by a two-tailed Mann–Whitney test) in LTα−/− mice than in WT mice (Fig. 3b). CCL2 is a major ligand for CCR2, which has other potential ligands, such as CCL7,
CCL8 and CCL12\textsuperscript{36,37}. Although CCL8 has been detected in the thymus\textsuperscript{39}, CCL7 and CCL12 have not been yet described to be expressed in this tissue. In contrast to CCL7, we found that CCL8 and CCL12 expression was also increased in Lta\textsuperscript{−/−} mTECs (Fig. 3c). Considering that LTA is induced upon crosstalk (Fig. 2a), we next examined whether CCL8, CCL12 and CCL12 expression in mTECs could be regulated by crosstalk with OTII CD\textsuperscript{4} thymocytes. The expression of three ligands was increased in mTECs from OTII:RipmOVA mice compared with OTII:OTII mice (Fig. 3d), which was even more pronounced in OTII:RipmOVA mice backcrossed on a Lta\textsuperscript{−/−} background, indicating that LTA represses mTEC ability to express these chemokines upon crosstalk. To further determine the role of direct Ag-specific interactions with CD4\textsuperscript{+} thymocytes in this chemokine expression, WT mTECs loaded or not with OVA\textsubscript{323-339} peptide were co-cultured with OTII CD\textsuperscript{4} thymocytes. CCL2, CCL8 and CCL12 were upregulated in OVA\textsubscript{323-339}-loaded mTECs compared with unloaded mTECs (Fig. 3e). Moreover, the addition of a soluble LT\textbeta R-Fc chimera, which blocks LTA\textbeta R2/LT\textbeta R2 interactions, resulted in more pronounced upregulation of these chemokines, indicating that LTA\textbeta R2/LT\textbeta R2 axis acts as a negative regulator of these chemokines upon mTEC-CCL7 and CCL12 crosstalk. We also found higher levels of CCL2, CCL8 and CCL12 in mTECs co-cultured with CD4\textsuperscript{+} thymocytes from OTIIxLta\textsuperscript{−/−} compared with those from OTII mice, suggesting that LTA, specifically in CD4\textsuperscript{+} thymocytes, controls the expression of these chemokines (Fig. 3f). Moreover, although DCs can cross-present Ags expressed by mTECs\textsuperscript{13,14}, the chemokine upregulation observed in co-cultures with OTII CD\textsuperscript{4} thymocytes, in absence of DCs (Fig. 3e), indicates that interactions with CD4\textsuperscript{+} thymocytes are sufficient to induce CCL2, CCL8 and CCL12 expression in mTECs. Using an antibody allowing the detection of MCP1\textalpha (i.e., CCL2, CCL7, CCL8 and CCL13, the latter being not expressed in mice), we found that in contrast to mTECs, thymic DC subsets did not express detectable levels of these chemokines in OTII-Rag\textsuperscript{2−/−} and RipmOVAxOTII-Rag\textsuperscript{2−/−} mice as well as in WT and Lta\textsuperscript{−/−} mice (Supplementary Fig. 9). This suggests that DCs do not possess the ability to attract peripheral APCs through the production of these chemokines. Finally, in contrast to OVA\textsubscript{323-339}-loaded SHPS-1\textsuperscript{+} cDCs, SHPS-1\textsuperscript{−} cDCs and pDCs, only OVA\textsubscript{323-339}-loaded mTECs were able to induce LTA expression in CD4\textsuperscript{+} thymocytes, excluding a potential implication of DCs in the regulation of these chemokines through LTA induction (Fig. 3g). Altogether, these data show that LTA represses CCL2, CCL8 and CCL12 expression induced in mTECs upon crosstalk with CD4\textsuperscript{+} thymocytes.

LTA-regulated thymic entry of APCs depends on CCR2. CCL2, CCL8 and CCL12 chemokines are known ligands for CCR2\textsuperscript{36}, and CCL8 is also a ligand for CCR1 and CCR5\textsuperscript{36,37,38,39}. To investigate a potential involvement of these chemokine receptors in the thymic entry of peripheral DCs and macrophages, we first examined their expression in blood-derived SHPS-1\textsuperscript{+} cDCs, pDCs and macrophages in WT and Lta\textsuperscript{−/−} mice. All these cell types significantly expressed CCR2 in these mice (p < 0.05 by two-tailed Mann–Whitney test, Supplementary Fig. 10), indicating that they possess the ability to migrate in a CCR2-dependent manner into the thymus. CCR1 and CCR5 were also weakly detectable in these three cell types, although macrophages had high levels of CCR1 in both mice. To determine CCR2, CCR1 and CCR5 contributions in regulating the thymic pool of SHPS-1\textsuperscript{+} cDCs, pDCs and macrophages, we generated mixed BM chimeras, in which lethally irradiated CD45.1xCD45.2 WT recipients were reconstituted with CD45.1 WT BM cells together with either CD45.2 WT, Ccr2\textsuperscript{RFP/RFP}, Ccr1\textsuperscript{−/−} or Ccr5\textsuperscript{−/−} BM cells (ratio 1:1) (Fig. 4a). Six weeks later, thymic SHPS-1\textsuperscript{+} cDCs, pDCs and macrophages were analysed (Supplementary Fig. 11). Strikingly, we found strongly reduced frequencies of these three cell types derived from CD45.2 Ccr2\textsuperscript{RFP/RFP} BM cells compared with those derived from CD45.2 WT BM cells (Fig. 4b). While macrophages were unaffected, we also found a slight reduction in SHPS-1\textsuperscript{+} cDCs and pDCs derived from CD45.2 Ccr1\textsuperscript{−/−} BM and in these three cell types derived from CD45.2 Ccr5\textsuperscript{−/−} BM cells. These results indicate that compared with CCR1 and CCR5, CCR2 is a key regulator of the thymic pool of SHPS-1\textsuperscript{+} cDCs, pDCs and macrophages.

Finally, to firmly demonstrate that enhanced thymic entry of DCs and macrophages in Lta\textsuperscript{−/−} mice was mediated by CCR2, blood nucleated cells from CD45.1 WT mice and Ccr2\textsuperscript{RFP/RFP} deficient mice (ratio 1:1) were co-transferred into sublethally irradiated WT and Lta\textsuperscript{−/−} recipients (Fig. 4c). Consistently, with our adoptive transfer (AT) experiments in Lta\textsuperscript{−/−} mice (Fig. 2e, f), frequencies and numbers of CD45.2 \textsuperscript{RFP} cells corresponding to CD45.1 donor cells in WT recipients (Fig. 12a) were more elevated in Lta\textsuperscript{−/−} than in WT recipients (Fig. 4d). Among total donor cells, we found increased frequencies and numbers of cDCs, pDCs and macrophages (Supplementary Fig. 12b, c). In contrast, increased thymus homing of total donor cells and of these three cell types in Lta\textsuperscript{−/−} mice was strongly impaired when donor cells were of Ccr2\textsuperscript{RFP/RFP} deficient origin (Fig. 4d, e). Altogether, these data reveal that LTA controls the thymus homing of APCs in a CCR2-dependent manner.

**Induction of CCR2 ligands and NF-κB subunits in Lta\textsuperscript{−/−} mice.** We next investigated by which mechanisms CCL2, CCL8 and CCL12 chemokines are overexpressed in Lta\textsuperscript{−/−} mTECs. This cell type can be subdivided into two main subsets based on CD80 level\textsuperscript{40} (Fig. 5a). Consistently with previous studies\textsuperscript{41,42}, Lta\textsuperscript{−/−} mice have normal frequencies and numbers of CD80\textsuperscript{lo} (mTEClo) and CD80\textsuperscript{hi} (mTEChi) mTECs, suggesting that increased expression in these chemokines (Fig. 3b, c) was not due to increased mTEC numbers. We found, by qPCR and flow cytometry, that CCR2 ligands were specifically upregulated in Lta\textsuperscript{−/−} mTEClo (Fig. 5b, c). CCL2 and CCL8 are known to be regulated by the classical NF-κB pathway in different cell types\textsuperscript{24–26}. Notably, p65 binding to the mouse Ccl2 promoter is involved in CCL2 expression\textsuperscript{43,44}. We identified two putative NF-κB binding sites for c-Rel and p65, by in silico analysis, in the Ccl2 promoter region (Supplementary Table 1), suggesting that this gene could be also regulated by the classical NF-κB pathway. The level of p65 phosphorylation at serine 536 (ser536), which is associated with the upregulation of CCL2\textsuperscript{45,46}, was unaltered in Lta\textsuperscript{−/−} mTEClo (Fig. 5d). We next assessed whether Lta\textsuperscript{−/−} mTEClo have a differential usage in the classical and non-classical NF-κB pathways, the latter known to be preferentially induced by LTA\textbeta R2/LT\textbeta R2 axis\textsuperscript{6,47}. We found at mRNA and protein levels that the non-classical NF-κB subunit Relb (RelB) was decreased whereas classical NF-κB subunits Rel (c-Rel) and Rela (p65) were enhanced in Lta\textsuperscript{−/−} mTEClo (Fig. 5e–g).

We next analysed the effect of LTA\textbeta R2/LT\textbeta R2 axis upon Ag-specific interactions with CD4\textsuperscript{+} thymocytes in the regulation of NF-κB subunits in mTEClo that express the LT\textbeta R receptor (Fig. 5h). Interestingly, mTECs co-cultured with OTIIxLta\textsuperscript{−/−} CD4\textsuperscript{+} thymocytes had reduced levels of Relb compared with mTECs co-cultured with OTII CD4\textsuperscript{+} thymocytes (Fig. 5i). In contrast, increased expression of Rel and Rela correlates with CCL2, CCL8 and CCL12 overexpression in these cells (Fig. 3f, Fig. 5j). Thus, the disruption of the LTA\textbeta R2/LT\textbeta R axis in the
context of Ag-specific interactions with CD4+ thymocytes leads to the upregulation of cRel and p65 classical NF-κB subunits and CCL2, CCL8 and CCL12 chemokines, suggesting that the chemokine upregulation in LTα−/− mTECs is controlled by the overexpression of classical NF-κB subunits.

**LTα-regulated APC thymic entry fine-tunes clonal deletion.** Since thymus homing of peripheral DCs and macrophages was enhanced in LTα−/− mice (Fig. 2e–h), we next investigated its impact on clonal deletion. Interestingly, numbers of DP (CD4+CD8+), CD4+CD8+ and CD4+ SP (CD4+CD8−) cells were significantly reduced in LTα−/− mice compared to WT mice (Fig. 6a, b). We also observed reduced numbers of CCR7− cortical and CCR7+ medullary CD4+ SP (Fig. 6c). This cannot be explained by defective thymus homing of early thymic progenitors, since we previously observed that their numbers were normal in LTα−/− thymi42. We thus analysed strongly auto-reactive thymocytes, based on the expression of the Ikaros family transcription factor Helios and PD-1, as previously described8.
(Fig. 6a). We found reduced numbers of autoreactive Helios+ PD-1− cells in CCR7− and CCR7+ CD4+ SP cells in Lta−/− mice, suggesting that clonal deletion was enhanced both in the cortex and medulla in these mice (Fig. 6d, e). Furthermore, we found reduced numbers of cortical Helios+PD-1−CD4+CD8+ post-positively selected cells (Fig. 6f). Similar results were observed when Lta−/− BM cells were transplanted into CD45.1 WT recipients (Supplementary Fig. 13), suggesting that Lta expression in haematopoietic cells controls the deletion of cortical and medullary thymocytes. An enhanced clonal deletion in Lta−/− mice is supported by reduced frequencies and numbers of CD69−CD62L+ mature CD4+ SP in these mice (Fig. 6g). Therefore, these data suggest that enhanced thymus homing of DCs and macrophages in Lta−/− mice leads to increased clonal deletion.

To confirm that Lta-regulated thymic entry of DCs and macrophages impacts the negative selection, OTII-Rag2−/− mice were backcrossed on a Lta−/− background. Similarly to Lta−/− mice (Fig. 3b, c), Ccl2, Ccl8 and Ccl12 were upregulated in mTECs from OTII-Rag2−/− x Lta−/− compared to OTII-Rag2−/− mice (Fig. 7a). Consistent with our findings that Lta regulates DC and macrophage thymic cellularity (Fig. 2b–d), numbers of SHPS-1+ cDCs, pDCs and macrophages were higher in the thymus of these mice (Fig. 7b). Furthermore, thymi of OTII-Rag2−/− x Lta−/− recipients previously transferred with CD45.1 DC and macrophage-enriched cells contained increased numbers of donor SHPS-1+ cDCs, pDCs and macrophages (Fig. 7c–e), confirming that the thymic migration of APCs is favoured on a Lta−/− background. Furthermore, we found that the AT of OVA323−/− loaded DC and macrophage-enriched cells was effective at eliminating OTII DP and Va2+ Vβ5+ CD4+ SP cells compared with unloaded APCs (Fig. 7f, g). This phenomenon was increased in OTII-Rag2−/− x Lta−/− mice (Fig. 7h), confirming an enhanced negative selection on a Lta−/− background. These data thus firmly demonstrate that Lta-regulated APC thymic entry controls the clonal deletion of autoreactive thymocytes.

**High ability of cDCs and macrophages for clonal deletion.** We next assessed the tissue distribution of SHPS-1+ cDCs, pDCs and macrophages by transferring cells sorted from CCR2RFP+/+ heterozygous mice (Fig. 8a), allowing us to track their thymic entry with the red fluorescent protein (RFP) reporter gene. For better detection, cell-sorted peripheral DCs and macrophages from CCR3RFP+/+ donor mice were adoptively transferred into Lta−/− recipients, in which thymus homing of these cells is increased (Fig. 2e–h). Consistent with our AT experiments of CD45.1 donor cells (Figs. 1e–h, Supplementary Fig. 14a, b), we found that these three cell types efficiently homed into the Lta−/− thymus and retained their phenotypic hallmarks, as revealed by CD11c, B2ST-2 and F4/80 staining (Fig. 8b). Interestingly, RFP+ cDCs and pDCs preferentially located in the cortex, whereas RFP+F4/80+ macrophages were similarly distributed in the cortex and medulla.

To investigate the respective role of these three cell types in clonal deletion, WT BM-derived cDCs, pDCs and macrophages, expressing CCR2 and MHCIi molecules, were generated (Fig. 8c, Supplementary Fig. 14c–e). This indicates that these cell types should be competent to migrate in a CCR2-dependent manner and present Ags via MHCIi molecules. The same number of OVA323−/− loaded BM-derived cDCs, pDCs or macrophages was first adoptively transferred into OTII-Rag2−/− mice (Fig. 8d). OVA323−/− loaded BM-derived cDCs and pDCs were able to delete autoreactive thymocytes in OTII-Rag2−/− mice compared to non-injected OTII-Rag2−/− controls (Fig. 8e). Whereas the role of thymic macrophages in clonal deletion remains largely elusive, we found that OVA323−/− loaded BM-derived macrophages were able to delete in vivo both DP and Va2+Vβ5+ CD4+ SP thymocytes (Fig. 8e). This is consistent with the observation that adoptively transferred macrophages were localised in both the cortex and the medulla (Fig. 8b), whereas DP and SP thymocytes were respectively eliminated. Importantly, BM-derived cDCs and macrophages were more efficient than BM-derived pDCs in deleting total thymocytes, including DP and Va2+ Vβ5+ CD4+ SP cells. Consistently, BM-derived cDCs and macrophages expressed higher levels of MHCIi than BM-derived pDCs (Fig. 8c). Furthermore, thymic SHPS-1+ cDCs express higher levels of MHCIi than pDCs, and are thus more prone to clonal deletion20. OVA323−/− loaded BM-derived cDCs, pDCs and macrophages deleted more efficiently total thymocytes, DP and Va2+Vβ5+CD4+ SP cells in OTII-Rag2−/−x Lta−/− than in OTII-Rag2−/− mice (Fig. 8e), which is consistent with the superior ability of these three cell types to home into the thymus on a Lta−/− background (Fig. 2e–h, Fig. 7d, e). Altogether these data indicate that migratory cDCs and macrophages have a higher capacity to delete autoreactive thymocytes than pDCs, a phenomenon accentuated in Lta−/− mice.

**Discussion**

Thymic clonal deletion crucially prevents the generation of hazardous autoreactive T cells that could induce autoimmunity. mTECs are essential in this process through their ability to widely express self-Ags that can be cross-presented by resident cDCs13,15,38,49. Peripheral DCs, by continuously migrating into the thymus, also contribute to the deletion of autoreactive thymocytes by sampling peripheral self-Ags17–19. Although migratory DCs are involved in this tolerogenic process, the implication of mTEC-thymocyte crosstalk in regulating their thymic recruitment remains unknown so far.

We provide strong evidence that Ag-specific interactions between mTECs and CD4+ thymocytes regulate the thymic entry of peripheral DCs and macrophages. We found increased numbers of SHPS-1+ cDCs, pDCs and macrophages in the
thymus of RipmOVAxOTII-Rag2−/− and OTII-RipmOVA mice compared with OTII-Rag2−/− and OTII:OTII mice, respectively. Furthermore, thymus homing of these cell types was enhanced in RipmOVAxOTII-Rag2−/− recipients upon AT of donor cells. Although RANKL and CD40L are implicated in mTEC-thymocyte crosstalk, 12, these two TNF members were expressed at similar extents in CD4+ thymocytes from OTII-Rag2−/− and RipmOVAxOTII-Rag2−/− mice, indicating that they are unlikely responsible for the increased thymic entry of peripheral APCs observed in RipmOVAxOTII-Rag2−/− mice. Nevertheless, we cannot exclude a potential role of these TNF members in other aspects of thymic DC biology. Future investigations are expected to clarify this issue. We show that this regulatory mechanism of peripheral APC recruitment is tightly controlled by LTA, which is specifically induced in autoreactive CD4+ thymocytes upon crosstalk with mTECs 50. Of note, LTA was shown to be expressed
Helios+ Lt-tailed Mann analysed for CD4 and CD8 expression. Foxp3–CD4loCD8lo cells were analysed for the expression of Helios and PD-1.

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**Fig. 6** Enhanced clonal deletion in the thymus of Lt-t/- mice. **a** Gating strategy used to analyse thymocyte subsets by flow cytometry. Foxp3– cells were analysed for CD4 and CD8 expression. Foxp3–CD4+SP (CD4+CD8–) cells were analysed for the expression of CCR7. CCR7–CD4+, CCR7+CD4+ SP and CD4loCD8lo cells were analysed for the expression of Helios and PD-1. **b** Flow cytometry profiles and numbers of DP, CD4loCD8lo and CD4+SP thymocytes (b) and CCR7–CD4+ and CCR7+CD4+ SP cells (c). **d-f** Flow cytometry profiles and numbers of Helios+PD-1-, Helios+PD-1+ and Helios–PD-1+ cells in CD4+CCR7– (d), CD4+CCR7+ (e) and CD4loCD8lo cells (f) in the thymus from WT and Lt-t/- mice. **g** Flow cytometry profiles, frequencies and numbers of CD69+CD62L– semi-mature and CD69–CD62L+ mature in Foxp3–CD4+ SP thymocytes from WT and Lt-t/- mice. **b-g** Data are representative of two independent experiments (n = 4 mice per group and per experiment). Error bars show mean ± SEM, *p < 0.05, **p < 0.01 using one-tailed Mann-Whitney test.
Fig. 7 LTα-regulated thymic entry of DCs and macrophages leads to an enhanced clonal deletion. a Ccl7, Ccl2, Ccl8 and Ccl12 mRNAs were measured by qPCR in purified mTECs (CD45+Ep-CAM<sup>+</sup>BP-1<sup>lo</sup>UEA-1<sup>+</sup>) from OTII-Rag2<sup>−/−</sup> (<em>n</em> = 3) and OTII-Rag2<sup>−/−</sup>×Ltα<sup>−/−</sup> (<em>n</em> = 3) mice. Data are representative from two independent experiment (<em>n</em> = 1–2 mice per group). b Numbers of SHPS-1<sup>+</sup> cDCs, pDCs and macrophages were analysed by flow cytometry in the thymus from OTII-Rag2<sup>−/−</sup> and OTII-Rag2<sup>−/−</sup>×Ltα<sup>−/−</sup> mice. c Experimental setup: AT of unloaded or OVA<sub>323–339</sub>-loaded DC and macrophage-enriched cells from CD45.1 WT mice. d, e Flow cytometry profiles, frequencies and numbers of DP (f) and Vβ<sup>5+</sup>Vα<sup>2+</sup>CD4<sup>+</sup> SP (g) cells analysed three days after AT. Data are representative from three to four independent experiments (<em>n</em> = 3–4 mice per group and per experiment). <em>b, c, d, f, g</em> MΦ: macrophage. Error bars show mean ± SEM, *<em>p</em> < 0.05, **<em>p</em> < 0.01, using one-tailed Mann–Whitney test.
in SP thymocytes as a membrane anchored LTα1β2 hetero-
complex, which binds to LTβR10,47. Our data show that LTα
negatively regulates the expression of CCL2, CCL8 and CCL12
chemokines in mTECs, which attenuates thymus homing of
APCs, mainly in a CCR2-dependent manner. Consequently,
migratory cDCs, pDCs and macrophages were increased in
Ltα−/− compared to WT thymi. Contrarily to Ltα−/− mice, Ltbr−/− mice have reduced numbers of thymic SHPS-1− resident
cDCs and pDCs51. It remains nevertheless unclear whether LTβR
controls the thymic pool of macrophages and peripheral APC
entry. These differences are not surprising, since these mice have
distinct defects in medulla organisation, mTEC subsets and autoimmunity12. Here, we demonstrate by AT of donor cells both in sublethally irradiated and unmanipulated mice, that LTα negatively regulates the thymus homing not only of peripheral
cDCs and pDCs but also of macrophages.

Our results, based on OTII:OTII and OTII:RipmOVA mTECs
and mTEC-CD4+ thymocyte co-cultures, strongly indicate that

![Diagram](image_url)
Ccl2, Ccl8 and Ccl12 were upregulated in mTECs upon Ag-specific interactions with CD4+ thymocytes. This upregulation was negatively controlled by LTA, specifically in CD4+ thymocytes, since it was exacerbated in absence of LTA or when LTA-1β2/LTβR interactions were blocked. Furthermore, CCL2, CCL8 and CCL12 were speciﬁcally upregulated in LTA+/−mTECs. Although mTEC36 were initially described to contain precursors giving rise to functional mTEC35,12, they were reported to contain mTECs, expressing the chemokine CCL21 and the transcription factor FEZF2 implicated in the attraction of thymocytes into the medulla and in the expression of self-Ags, respectively52,53. Our study revealed that mTEC36 also express chemokines involved in the thymic entry of peripheral APCs, further highlighting a functional role of this subset in T cell selection. We further show that LTA controls mTEC36 properties by regulating CCL2, CCL8 and CCL12. This chemokine upregulation was likely due to a differential usage in the classical and non-classical NF-κB pathways, which are known to function as an interconnected signalling network rather than two independent pathways, even in mTECs6,54,55. Whereas in WT conditions, the lymphotixin signalling activates preferentially the non-classical NF-κB pathway6,47, our data indicate a preferential usage of the classical NF-κB pathway, characterised by an upregulation of cRel and p65 when the lymphotixin signalling is disrupted. Since CCL2 and CCL8 are regulated by classical NF-κB members24–26 which are overexpressed in LTA+/−mice, this chemokine upregulation observed in LTA+/−mTECs is likely mediated by the classical NF-κB pathway. Chromatin immunoprecipitation assays are expected to prove a direct regulation of these chemokines by classical NF-κB members. However, the mTEC36 subset represents a rare cell type (~2 × 103 cells/thymus) rendering technically difficult such an approach. Furthermore, although DCs can cross-present Ags expressed by mTECs33,14, it is unlikely that they participate in CCL2, CCL8 and CCL12 expression since cultures of CD4+ thymocytes with mTECs alone was sufﬁcient to induce these chemokines. Furthermore, DCs did not express CCR2 ligands in the context of mTEC-CD4+ thymocyte crosstalk or upon LTA-mediated regulation. Another hypothesis would be that DCs could be indirectly involved in this regulation by inducing LTA in CD4+ thymocytes. We showed that in contrast to mTECs, neither thymic SHPS-1−, SHPS-1− cDCs nor pDCs were able to induce LTA in CD4+ thymocytes, excluding a potential contribution of DCs in the expression of these chemokines through LTA upregulation. Nevertheless, we cannot deﬁnitely rule out a role of DC-mediated indirect antigen presentation in the thymic recruitment of peripheral APCs. Experiments based on the use of DC-depleted mice in the context of thymic crosstalk are expected to clarify this issue in the next future.

Mixed BM chimera and AT experiments demonstrated that CCR2 plays a major role in the thymic homing of SHPS-1+cDCs, pDCs and macrophages. Although a drastic reduction (~80–95%) of these three cell types was observed in the Ccr2−/−/RFP−/mice, a moderate but non-negligible decrease of thymic SHPS-1+cDCs, pDCs and macrophages was observed in Ccr1−/− and Ccr5−/− groups. While pDCs have been reported to migrate through CCR910, our data thus show that they also use CCR2. Moreover, co-AT of WT and Ccr2−/−/RFP−/deficient donor cells indicates that although the thymic entry of SHPS-1+cDCs is impaired in the absence of CCR2, this process is not fully abrogated, suggesting the implication of other chemokine receptor(s). Potential candidates could be CCR1 and CCR5, since thymic SHPS-1− cDCs were moderately disturbed in Ccr1−/− and Ccr5−/− donor groups. Future experiments, based on the analysis of Ccr2 and Ccr5 as well as Ccr2 and Ccr1 double-deﬁcient mice, are expected to clarify this issue.

Interestingly, since negatively selected thymocytes do not directly die, but instead remain viable for few hours in the medulla36, it is likely that autoreactive thymocytes have sufﬁcient time to provide instructive signals to mTECs, that would regulate the thymic recruitment of peripheral DCs and macrophages. Interestingly, we demonstrate that this regulation loop controls the clonal deletion of autoreactive T cells (Supplementary Fig. 15). Autoreactive thymocytes were highly deleted at the DP, CD40CD80 and CD4+ SP stages in LTA+/− mice, indicating that enhanced clonal deletion occurs in both the cortex and medulla. By AT experiments, we demonstrated that cDCs, pDCs and macrophages were all able to delete efﬁciently DP and SP cells, a process accentuated on a LTA+/− background. According to the type of negatively selected cells, SHPS-1− cDCs and pDCs were preferentially localised in the cortex, whereas macrophages were distributed throughout the thymus. Macrophages have been associated with the clearance of apoptotic bodies in the thymus22,23. Although they share common hallmarks with DCs, by expressing MHCII and CD80/CD86 molecules, implicated in T cell selection, their role in clonal deletion and the mechanisms that sustain their thymic entry remain unknown. So far, only one group has reported that F4/80+CD11b+ macrophages are able to delete autoreactive thymocytes in vitro by using reagggregated thymic organ culture57. Here, we unravel that peripheral macrophages migrate in a CCR2-dependent manner into the thymus and that they may play an unsuspected role in clonal deletion.

Finally, from a therapeutic perspective, because LTA deﬁciency increases DC and macrophage thymic entry, it would be interesting to determine whether LTA loss can protect and treat from autoimmunity. Generating an inducible transgenic mouse model allowing LTA deletion at a speciﬁc time point, i.e., before or after the development of autoimmune signs, would be useful to deﬁne the potential of LTA as a new target to prevent or treat autoimmunity associated with defective T cell selection.

In sum, this complex cellular interplay between mTECs, CD4+ thymocytes, peripheral DCs and macrophages constitutes a fine..
tuning mechanism that allows the thymus to adapt its capacity of deleting autoreactive T cells to physiological and pathological fluctuations. This study should open new therapeutic perspectives for autoimmune disorders, based on the deletion of hazardous T cells via the manipulation of thymic entry of peripheral DCs and macrophages.

**Methods**

***Mice.*** CD45.1 WT (B6;S1-Ptp46.6Pic6.5BoyCrl, Stock n°002014, Charles River), CD45.2 WT (Stock n°000664, Charles River), CD45.2 Lta−/−, 14, CD45.2 Ccr2−/−, fluororescent protein (RFP) (RFPCCR2RFP39), CD45.2 Cer−/−, 60, CD45.2 Cer−/−, 61, OTII− and RipmOVA− mice were on a C57BL/6J background. OTII and OTII− mice were backcrossed on a C57BL/6J background (C57BL/6j/c/Ha.129P2-3wP1-BoyCrl, Stock n°002014, Charles River). Male and female mice were used at the age of 6 weeks. Mice were killed using CO2 and then terminated via cervical dislocation. All experiments were done in accordance with national and European laws for laboratory animal welfare (EEC Council Directive 2010/63/UE), and were approved by the Marseille Ethical Committee for Animal Experimentation (Comité National de Référence Ethique sur l’Expérimentation Animale no. 14).

**Bone marrow chimeras.** After flushing with RPMI medium, 5 × 10^8 BM cells from tibia and femurs of OTII-Rag2−/− mice were injected i.v. into lethally irradiated OTII-Rag2−/− and RipmOVA-Rag2−/− recipients (two doses of 500 rads, 5 h apart) (Protocat, Radioisotopes). The same protocol was performed by transplanting 5 × 10^8 BM cells from CD45.2 WT or Lta−/− mice into lethally γ-irradiated CD45.1 WT recipients. For mixed bone marrow chimeras, 3 × 10^8 BM cells from either CD45.1 WT mice were co-injected i.v. with 3 × 10^8 BM cells from CD45.2 WT, Cer+/-/+RFP+, Cer−/− or Cer−/− mice into lethally γ-irradiated CD45.2 WT recipients. Chimeras were generated at 6–8 weeks of age. All BM transplants were performed with sex and age-matched mice.

**Cell isolation.** Thymus, spleen and lymph nodes were digested for 15 min at 37 °C in HBSS medium containing 1 mg/ml of collagenase D and 100 μg/ml of DNase I (Roche), and were subjected to vigorous pipetting. This step was repeated until complete tissue digestion. Cells were filtered through a 70 μm mesh to remove clumps. Splenic and lymphoid cells were lysed with RBC lysis buffer (eBioscience). Total mTECs were purified by sorting CD45 Ep-Cam−BP−1U-EA−1 cells after depletion of CD45 haematopoietic cells, using anti-CD45 magnetic beads by autoMACS with the DepleteS program (Miltenyi Biotec). mTEClo and mTEC hi cells were sorted from the thymus of WT mice, by sorting of SHPS-1+CD11chIBST-2lo and CD11intBST-2hi, respectively. Flow cytometry gating strategies are shown in Supplementary Fig. 1. CD4 + T cells were sorted from the thymus of WT mice, by sorting of CD45.1 WT congenic mice or CD3−CD19− antibodies with anti-biotin microbeads by AutoMACS via the deplete program (Miltenyi Biotec). All 5 × 10^6 DC and macrophage-enriched cells, spleens and lymph nodes from CD45.1 WT congenic mice or Cer+/-/+RFP+ mice were depleted in T and B cells, using biotinylated anti-CD3 and anti-CD19 antibodies with anti-biotin microbeads by AutoMACS via the deplete program (Miltenyi Biotec). For co-adoptive transfer experiments, red blood cells from CD45.1 WT congenic mice or Cer+/-/+RFP+ homologous mice were lysed with RBC lysis buffer (eBioscience). All 3 × 10^6 nucleated blood cells from CD45.1 WT mice were injected i.v. into lethally irradiated OTII− mice and sorted by FACS and FlowJo software (BD). Analysis was performed with a FACSCanto II (BD) and data were analysed using Flowjo software (BD).

**In vitro co-culture assays.** All 2 × 10^5 cell sorted-CD45 Ep-Cam−BP−1UEA−1 + mTECs, SHPS-1CD11cIBST-2lo dCps, SHPS-1CD11cIBST-2hi dCps and CD11dIBST-2hi dPCs from WT thymi were loaded or not for 1 h with OVA323-339 peptide (10 ng per ml Polypeptide group) at 37 °C. Cells were then cultured for 24 h with 1 × 10^5 purified CD44+ thymocytes, from OTII-Rag2−/− or OTII-Rag2−/−/lntx−/− mice, in a medium containing RPMI (ThermoFisher) and 10% FBS (Sigma Aldrich) supplemented with L-glutamine (2 mM, ThermoFisher), sodium pyruvate (1 mM, ThermoFisher), 2-mercaptoethanol (2 × 10−5 M, ThermoFisher), penicillin (100 IU per ml, TherMoFisher) and streptomycin (100 μg per ml, ThermoFisher). When indicated, recombiant LTβR-Fc chimera (2 μg per ml, RnD systems) was added to the culture medium.

**Adoptive transfer of blood and splenic CD45.1 donor cells.** Red blood cells from CD45.1 WT congenic mice or Cer+/-/+RFP+ homologous mice were lysed with RBC lysis buffer (eBioscience). All 3 × 10^6 nucleated blood cells from CD45.1 WT congenic mice or Cer+/-/+RFP+ homologous mice were injected i.v. into lethally and T cell depleting OTII− mice. OTII−/OVAxOTII Rag2−/−, CD45.2 WT or Lta−/− recipients (one dose of 500 rads; X-ray; RS-2000 Irradiator; Rad Source Technologies). For co-adoptive transfer experiments, OTII−/OVAxOTII Rag2−/−, CD45.2 WT or Lta−/− recipients (one dose of 500 rads; X-ray; RS-2000 Irradiator; Rad Source Technologies). For AT of DC and macrophage-enriched cells, spleens and lymph nodes from CD45.1 WT congenic mice or Cer+/-/+RFP+ homologous mice were depleted in T and B cells, using biotinylated anti-CD3 and anti-CD19 antibodies with anti-biotin microbeads by AutoMACS via the deplete program (Miltenyi Biotec).

**Bone marrow-derived cDCs/pDCs/macrophages.** After red blood cell lysis (eBioscience), BM cells from WT mice were cultured for 7–10 days in complete RPMI medium containing M-CSF (30 ng per ml, RnD systems), murine Flt3-ligand (100 ng per ml, PeproTech) and GM-CSF (20 ng per ml, PeproTech) for the generation of cDCs, pDCs and macrophages, respectively. Cellular differentiation was assessed by flow cytometry.

**Immunofluorescence staining.** Thymi were harvested and frozen in O.C.T. (Sakura Finetek). The 20-μm sections were fixed with 2% paraformaldehyde (Sigma Aldrich) and permeabilized by 0.1% Triton-X. The following antibodies were detected: Alexa Fluor 488-conjugated anti-F4/80 (10F12, 1 μg per ml, ThermoFisher), mouse IgG anti-CD45.2 (eBioscience). All antibodies and Alexa Fluor 488-conjugated anti-rabbit IgG or Alexa Fluor 488-conjugated anti-rabbit IgG (Invitrogen), respectively. Sections were counterstained with DAPI (BioLegend) and mounted with Mowiol (Calbiochem). Immunofluorescence confocal microscopy was performed with a LSM 780 Leica SP5X confocal microscope.

**Confocal image analysis.** Confocal microscopy images were segmented using homemade Matlab scripts. Twenty-five images for Lta−/− thymic sections with AT of purified DCs and macrophages from Cer+/-/+ mice and six to eleven images for WT and Lta−/− thymic sections were analysed. Briefly, images were sequentially treated by: (i) median filtering over 2 pixels, (ii) thresholding, using the automatic threshold value (according to Otsu method, as implemented in the Matlab function graythresh), (iii) smoothing (dilation followed by erosion) over 3 pixels, and (iv) background subtraction, with the background estimated by median filtering of the colocalization image over 32 pixels. For cells expressing RFP, colocalization between the green (endogenous DC or macrophage staining) and red channels (RFP) was estimated as the minimum intensity of the images of both channels. This ensured validating signals arising only from high staining for both color, thus mimicking self-recognition. In all cases, the intensity of the resulting binary images. The median was detected from the K14 staining by median filtering over 7 pixels, automatically thresholding, filling holes and smoothing over 30 pixels. Defining a binary mask corresponding to the median and the cortex allowed automated counting of cells in each compartment. Cell density was computed as cell number over volume (S − N/S), for median and cortex, allowing computing the ratio of medulla versus cortex density (ratio = d_medulla/d_cortex).
Quantitative RT-PCR. Total RNA was prepared with TRIsol reagent (Invitrogen). cDNA was synthesized with oligo dT primers (Life Technologies) and Superscript II reverse transcriptase (Invitrogen). PCR was performed using SYBR Premix Ex Taq (Takara) with the ABI Prism 7500 Fast PCR System (Applied Biosystems). Actin mRNA was used for normalisation. Primers are listed in Supplementary Table 3.

Analysis for NF-κB binding sites in the Ccl12 promoter. In silico analysis of the mouse Ccl12 promoter was performed using the software MatInspector (https://www.genomatix.de). The promoter region from −1000 to +100 bp of the transcription start site was searched for NF-κB binding sites using the Matrix family library version 10.0.

Statistics. All data are presented as means ± standard error of mean (SEM). Statistical analysis was performed with GraphPad Prism 7.03 software using unpaired Student’s t-test for normal distribution or Mann-Whitney test. ****p < 0.0001; **p < 0.01; *p < 0.05. Normal distribution of the data was assessed using d’Agostino-Pearson omnibus normality test.

Data availability. The authors declare that the data supporting the findings of this study are available within the article and its supplementary information files, or are available upon reasonable requests to the authors.

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Author contributions

N.L., J.C., O.C. and M.I. performed experiments and analysed the data. A.S. analysed the data. N.L. and M.I. interpreted data and wrote the manuscript. M.I. initiated, supervised and conceived the study.

Additional information

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