Administration of RANKL boosts thymic regeneration upon bone marrow transplantation

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Abstract

Cytoablative treatments lead to severe damages on thymic epithelial cells (TECs), which result in delayed de novo thymopoiesis and a prolonged period of T-cell immunodeficiency. Understanding the mechanisms that govern thymic regeneration is of paramount interest for the recovery of a functional immune system notably after bone marrow transplantation (BMT). Here, we show that RANK ligand (RANKL) is upregulated in CD4⁺ thymocytes and lymphoid tissue inducer (LTI) cells during the early phase of thymic regeneration. Importantly, whereas RANKL neutralization alters TEC recovery after irradiation, ex vivo RANKL administration during BMT boosts the regeneration of TEC subsets including thymic epithelial progenitor-enriched cells, thymus-homing of lymphoid progenitors, and de novo thymopoiesis. RANKL increases specifically in LTI cells, lymphotixin α, which is critical for thymic regeneration. RANKL treatment, dependent on lymphotixin α, is beneficial upon BMT in young and aged individuals. This study thus indicates that RANKL may be clinically useful to improve T-cell function recovery after BMT by controlling multiple facets of thymic regeneration.

Keywords bone marrow transplantation; lymphotixin α; receptor activator of nuclear factor kappa B ligand; T-cell reconstitution; thymic regeneration

Subject Category Immunology

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Introduction

The thymus controls the generation of a diverse repertoire of T lymphocytes. Cortical thymic epithelial cells (cTECs) support the differentiation of T-cell progenitors and the conversion of CD4⁺ CD8⁺ double-positive (DP) thymocytes into CD4⁺ CD8⁻ and CD4⁻ CD8⁺ single-positive (SP) cells (Anderson & Takahama, 2012). Medullary TECs (mTECs) purge the TCR repertoire of hazardous autoreactive T cells by expressing thousands of tissue-restricted antigens (TRAs), which are controlled by Aire (Autoimmune Regulator) and Fez2 (Forebrain Expressed Zinc Finger 2) factors (Derbinski et al, 2001; Anderson et al, 2002; Sansom et al, 2014; Takaba et al, 2015). Reciprocally, thymocytes sustain TEC differentiation and organization. This complex interplay is referred to as thymic cross talk (van Ewijk et al, 1994; Lopes et al, 2015).

Cytoablative treatments such as radiation or chemotherapy, used to prepare patients notably to bone marrow (BM) transplantation (BMT), severely affect not only hematopoietic cells but also TECs, which results in delayed T-cell reconstitution (Adkins et al, 1988; van den Brink et al, 2004; Fletcher et al, 2009; Hollander et al, 2010). Thymic injury triggered by total body irradiation (TBI) leads to profound alterations characterized by a drastic reduction in the cortex resulting from the massive depletion of DP thymocytes and a significant decrease in the medulla (Irla et al, 2013). Alterations in TEC ultrastructure and a reduction in some stromal cells have also been reported (Adkins et al, 1988; Irifune et al, 2004).

The recovery of a fully competent T-cell compartment is therefore a prolonged process that is considerably delayed compared to that of myeloid, NK or B cells (Hakim et al, 1997). This period of compromised immunity is prompt to serious clinical consequences such as opportunistic infections, autoimmunity, or tumor relapse and could lead to post-transplant morbidity and mortality (Curtis et al, 1997; Small et al, 1999; van den Brink et al, 2004; King et al, 2004; Parkman et al, 2006). Identifying an effective treatment that acts at several levels by improving (i) the regeneration of thymic epithelial progenitor cells (TEPCs), which are critical for the renewal of stromal niches; (ii) the recovery of cTECs and mTECs, which control the different steps of thymopoiesis; and (iii) thymus homing of T-cell progenitors, which is important for T-cell recovery, is of paramount clinical interest to optimally boost thymic regeneration (Penit & Ezine, 1989; Chen et al, 2004; Zlotoff et al, 2011).

RANK ligand, a TNF family member, has emerged as an important regulator of epithelial cell growth and differentiation in different tissues such as mammary glands during pregnancy (Fata et al, 2000), the renewal and epidermal growth of the hair follicles (Duheron et al, 2011), or M-cell differentiation from intestinal epithelial cells in Payer’s patches (Knoo et al, 2009). In the embryonic thymus, RANKL provided by a subset of innate lymphoid cells, namely lymphoid tissue inducers (LTI) cells, and invariant Vγ5⁺ TCR⁺ T-cell progenitors promotes the emergence of Aire⁺ mTECs, which mediate T-cell tolerance (Anderson et al, 2002; Rossi et al, 2007; Roberts et al, 2012). In the postnatal thymus, RANKL

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produced by SP thymocytes enhances numbers of Aire− mTECs and the size of the medulla (Akiyama et al., 2008; Hikosaka et al., 2008; Irla et al., 2008, 2010; Ohigashi et al., 2011; Lopes et al., 2015). However, although RANKL is a potent inducer of mTEC differentiation at steady state, whether and how RANKL drives thymic regeneration upon BMT remain unknown.

Here, we show that RANKL is upregulated early after thymic damage in CD4+ thymocytes and LTi cells. Importantly, whereas the in vivo neutralization of RANKL alters TEC regeneration after TBI, the ex vivo administration of RANKL substantially enhances the cellularity of cTEC and mTEC subsets as well as TEPC-enriched cells. Furthermore, we show that RANKL treatment induces lymphotxin α (LTα) upregulation specifically in LTi cells, which express its cognate receptor, RANK. Although at steady state LTα− mice show normal TEC subsets, Aire+ mTEC differentiation and T-cell development (De Togni et al., 1994; Venanzi et al., 2007; Seach et al., 2008), we demonstrate that LTα is critical for TEC regeneration and T-cell reconstitution. Importantly, RANKL administration during the early phase of BMT boosts the regeneration of TEPCs and TEC subsets, ameliorates T-cell progenitor homing and de novo thymopoiesis, which enhances peripheral T-cell reconstitution. Furthermore, we show that the effects mediated by RANKL depend on LTα expression and are also beneficial upon BMT in mice with early thymic involution. Altogether, our findings identify that the administration of RANKL constitutes a new therapeutic strategy to boost thymic regeneration upon BMT by acting at several levels: TEC recovery, T-cell progenitor homing, and de novo thymopoiesis.

**Results**

**RANKL is upregulated during the early phase of thymic regeneration**

Because at steady state RANKL has been reported as a potent regulator of mTEC differentiation (Rossi et al., 2007; Hikosaka et al., 2008; Ohigashi et al., 2011), we investigated whether this cytokine plays a role in thymic regeneration. To this, we first analyzed RANKL expression in the thymus at day 3 after SL-TBI (d3 SL-TBI) and found that RANKL was substantially upregulated in CD45+ hematopoietic cells compared to untreated (UT) WT mice (Fig 1A). We next investigated the cell identity of hematopoietic cells that overexpressed RANKL. We found that recipient CD4+ thymocytes and LTi cells, both previously described to be partially radio-resistant (Ueno et al., 2004; Dudakov et al., 2012; Fig 1B and C, Table 1, and Appendix Fig S1), upregulated RANKL at d3 SL-TBI (Fig 1D). Strikingly, although LTi cells represent a small subset in the thymus, they expressed higher levels of RANKL than CD4+ thymocytes after irradiation (Fig 1E). These results indicate that CD4+ thymocytes are crucial for RANKL upregulation after TBI, which in line with their high numbers after irradiation (Table 1). Since ZAP-70−/− mice have normal DP cells, these results also indicate that DP cells are not involved in RANKL upregulation. Given that LTi cells expressed high levels of RANKL after irradiation (Fig 1D), we decided to further define the contribution of this cell type in RANKL expression by analyzing the thymus from Rorc−/− mice, defective in LTi cells (Sun et al., 2000; Eberl et al., 2004). Irradiated Rorc−/− mice failed to increase RANKl, suggesting that this cell type was implicated in RANKL upregulation (Fig 1E). However, Rorc−/− mice were described to show reduced numbers of CD4+ thymocytes (Sun et al., 2000). In order to clearly determine the contribution of LTi cells, we then analyzed RANKL expression in the thymus of Rag2−/− mice, showing an early block in T-cell development at the double-negative DN3 stage but exhibiting LTi cells (Shinkai et al., 1992). At d3 SL-TBI, Rankl mRNA was upregulated in the Rag2−/− thymus but at lesser extent than in WT thymus, confirming that LTi cells also contribute to RANKL overexpression after TBI (Fig 1E). Interestingly, RANKL was upregulated in CD4+ SP and LTi cells until day 10 after SL-TBI with no hematopoietic rescue (Fig 1F). Of note, LTi cell ability to produce high level of RANKL in response to SL-TBI was much more pronounced than that of CD4+ thymocytes. Altogether, these data indicate that RANKL is naturally upregulated in both CD4+ SP and LTi cells at the early phase of thymic regeneration.

**RANKL neutralization inhibits TEC regeneration whereas ex vivo RANKL administration boosts TEC recovery after irradiation**

The aforementioned data strongly suggest that RANKL could play a role in thymic regeneration after irradiation. To confirm this assumption, WT mice were treated with a neutralizing anti-RANKL antibody (IK22/5) during 3 days after SL-TBI. PBS- and isotype antibody-treated mice were used as controls. RANKL neutralization was sufficient to prevent TEC regeneration illustrated by a 2.5-fold decrease in numbers of total TECs (EpCAM+), cTECs (EpCAM−UEA-1−Ly51−), and mTECs (EpCAM−UEA-1−Ly51−) compared to controls (Fig 2A). In addition, RANKL neutralization resulted in a decrease in CD80+CD80+EpCAM−Ly51+ and CD80+EpCAM−Ly51+ mTECs as well as several TEC subsets identified by MHCII expression level (Wong et al., 2014), including cTEC hi (MHCII+UEA-1−), mTEC hi (MHCII+UEA-1−), and mTEC lo (MHCII+UEA-1−) (Fig 2A and B). Interestingly, a TEC population described to be enriched in TEPCs defined as α6-integrin+Sca-1hi in the TEClo (MHCII+UEA-1lo) subset (Wong et al., 2014) was also decreased (Fig 2C). In a therapeutic perspective, we next investigated whether conversely the ex vivo administration of RANKL protein could improve TEC regeneration. WT mice were treated with RANKL-GST protein during 3 days after SL-TBI. PBS- and GST-treated mice were used as controls. Remarkably, RANKL-treated mice showed a 2-fold increase in numbers of total TECs, cTECs, and mTECs compared to controls (Fig 2A). RANKL treatment also enhanced CD80+EpCAM− and CD80+EpCAM− mTECs as well as cTEC hi, mTEC lo, TEClo, mTEC hi, and CD80+EpCAM− mTECs (Fig 2A and B). In a therapeutic perspective, we next investigated whether conversely the ex vivo administration of RANKL protein could improve TEC regeneration. WT mice were treated with RANKL-GST protein during 3 days after SL-TBI. PBS- and GST-treated mice were used as controls. Remarkably, RANKL-treated mice showed a 2-fold increase in numbers of total TECs, cTECs, and mTECs compared to controls (Fig 2A). RANKL treatment also enhanced CD80+EpCAM− and CD80+EpCAM− mTECs as well as cTEC hi, mTEC lo, TEClo, mTEC hi, and TEC hi-enriched cells. Numbers of proliferating Ki-67+ cells in these three subsets were decreased after RANKL neutralization, whereas they were increased after ex vivo RANKL administration (Fig 2D). Interestingly, the analysis of purified mTECs from RANKL-treated mice showed reduced expression of Bax, Bid, and Bak pro-apoptotic...
genes and that of cTECs exhibited reduced expression of Bax as well as an increased expression of the Bcl-xl anti-apoptotic gene (Fig 2E). Furthermore, the density of medullary Aire" cells and the expression of Aire and Aire-dependent TRAs were also enhanced in response to RANKL (Fig 2F and G). Of note, we also found that RANKL stimulated in cTECs the expression of Selp, Icam-1, and
Table 1. Cell numbers of lymphoid cells observed in the thymus of WT mice before and after d3 SL-TBI.

| Cell types | Untreated | d3 SL-TBI | P-values | Category |
|------------|-----------|-----------|----------|----------|
| DN (>10⁶)  | 15.86 ± 2.62 | 0.49 ± 0.07 | < 0.0001 | ***** |
| DP (>10⁴)  | 130.70 ± 14.46 | 0.27 ± 0.07 | < 0.0001 | ***** |
| CD4⁺ SP (>10⁵) | 18.01 ± 2.07 | 2.61 ± 0.20 | < 0.0001 | ***** |
| CD8⁺ SP (>10⁶) | 5.02 ± 0.79 | 0.68 ± 0.05 | < 0.0001 | ***** |
| Foxp3⁺ Tregs (>10⁴) | 3.5 ± 0.33 | 0.14 ± 0.01 | < 0.0001 | ***** |
| LTi cells (>10⁶) | 3.35 ± 0.72 | 1.58 ± 0.12 | 0.0021 | ** |

Average ± SD (Untreated: n = 10 thymi and d3SL-TBI: n = 18 thymi). P-values were calculated using Student’s t-test. **p < 0.01; *****p < 0.0001.

**Ccl21**, implicated in the thymus homing of lymphoid progenitors (Fig 2H). Altogether, these data point out that RANKL controls endogenous TEC recovery and that *ex vivo* RANKL administration boosts TEC regeneration by enhancing their proliferation, survival, and differentiation after thymic damage.

**RANKL controls LTα expression specifically in LTi cells after TBI**

We next investigated deeper the underlying mechanism(s) of RANKL treatment in the thymus after TBI. During embryogenesis, *in vitro* experiments have shown that RANKL induces LTα expression in peripheral LTi cells (Yoshida *et al*, 2002). A possible mechanism is thus that RANKL acts on this cell type. Interestingly, we found that irradiation led to the upregulation of RANKL cognate receptor, RANK on LTi cells (Fig 3A). Furthermore, to assess whether RANKL regulates LTα production in thymic LTi cells after irradiation, hematopoietic cells from irradiated WT thymi were stimulated *in vitro* with either RANKL-GST or GST. RANKL stimulation significantly upregulated LTα expression in LTi cells, while the addition of RANKL antagonist, RANK-Fc, fully abolished LTα induction, demonstrating the specificity of the treatment used (Fig EV1A). It is notable that among radio-resistant hematopoietic cells, only LTi cells upregulated LTα protein (Fig EV1B). In line with this, *in vivo* administration of RANKL during 3 days after SL-TBI also induced LTα upregulation selectively in thymic LTi cells (Fig 3B and C). Conversely, the administration of a neutralizing anti-RANKL antibody inhibited LTα upregulation only in LTi cells (Fig 3D and E). These data thus indicate that RANKL controls LTα upregulation specifically in LTi cells after SL-TBI. Moreover, LTi-deficient Rorc−/− thymus failed to increase LTα mRNA at d3 SL-TBI, suggesting that LTi cells are required for LTα overexpression after irradiation (Fig 3F). In contrast, LTα upregulation was maintained at WT level in the irradiated ZAP-70−/− thymus, lacking SP cells, indicating that radio-resistant CD4⁺ thymocytes are not involved in LTα upregulation after SL-TBI (Fig 3F). Since DP thymocytes constitute a non-negligible number after irradiation even if they are massively eliminated (Table 1) and that ZAP-70−/− mice have normal DP cells, we analyzed their potential contribution in LTα upregulation using Rag2−/− mice (lacking DP and SP cells). Irradiated Rag2−/− thymus expressed LTα at a similar level than that of irradiated WT mice, indicating that DP cells are not involved in LTα expression after SL-TBI and confirming that LTi cells are the main providers (Fig 3G). Furthermore, the thymus of irradiated WT mice deprived before of DP cells by dexamethasone treatment (Purton *et al*, 2004), also exhibited the same LTα expression level than that observed in irradiated WT and Rag2−/− mice (Fig 3G), confirming that LTα upregulation after SL-TBI does not rely on DP thymocytes. These data thus demonstrate that LTi cells are critical for LTα upregulation after irradiation.

Interestingly, LTα overexpression in LTi cells tightly correlated with that of RANKL during the course of BMT (Fig 3H). We found that LTα protein was selectively upregulated in LTi cells from recipient and not from donor origin until day 6 after BMT (Fig 3I and J), showing the importance of the host LTi cells in LTα production. Moreover, RANKL was expressed at normal level at d3 SL-TBI in LTα−/− mice, suggesting that LTα did not regulate RANKL (Fig 3K). We further observed that both Ltx and Ltβ mRNAs were increased in the total thymus at d3 SL-TBI compared to UT WT mice (Fig 3L) and that LTα protein was specifically induced in hematopoietic cells (Fig 3M). We thus hypothesized that LTα could be expressed as a membrane anchored LTαβ heterocomplex, which only binds to LTβR (Gommerman & Browning, 2003). We used a soluble LTβR-Fc fusion protein, which detects the two LTβR ligands, LTαβ2 and LIGHT. In contrast to Ltx and Ltβ, LIGHT mRNA was slightly expressed and not upregulated after thymic injury (Appendix Fig S2), indicating that LTβR-Fc staining detects only LTαβ2 in LTi cells, which was upregulated in a radiation dose-dependent manner (Fig 3N and O). These data thus show that RANKL treatment induces LTαβ upregulation specifically in LTi cells early after thymic injury.

**LTα is critical for TEC regeneration and *de novo* thymopoiesis during the course of BMT**

We next addressed whether LTα upregulation in response to RANKL treatment after TBI is involved in thymic regeneration. In line with this assumption, total TECs, cTECs, and mTECs but also TEPC-enriched cells upregulated LTβ receptor (LTβR) at d3 SL-TBI (Fig 4A). While at steady state, LTα−/− mice, in which the expression of LTαβ2 is fully lost, did not show any defect in TEC subsets (Fig 4B–F; Venanzi *et al*, 2007), numbers of cTECs, mTECs, and mTEC subsets (CD80⁺Aire⁺, CD80⁻Aire⁺, and CD80⁻Aire⁻) as well as the density of Aire⁺ cells were dramatically reduced at d3 SL-TBI (Fig EV2A–C). Of note, no significant defect in CD45⁺PDGFRα⁺ fibroblasts or in thymic LTi cells was observed in LTα−/− mice at d3 SL-TBI (Fig EV2D and E). To definitively address the role of LTα during thymic recovery after BMT, lethally irradiated CD45.2 WT or LTα−/− recipients were reconstituted with CD45.1 congenic BM cells (WT CD45.1:WT or WT CD45.1:LTα−/− mice) and TEC numbers were analyzed at days 10, 21, and 65 after BMT (Fig 4B–F). We observed reduced numbers of total TECs, cTECs, and mTECs as well...
Figure 2. RANKL is crucially involved in TEC regeneration after TBI.

A Flow cytometry profiles and numbers of total TECs (EpCAM+), cTECs (UEA-1+/Ly51+), and mTECs (UEA-1+/Ly51-) analyzed in CD45+ cells from UT WT mice or treated with PBS, a RANKL isotype control antibody (Iso.), a neutralizing anti-RANKL antibody (IK22/5), GST or RANKL proteins during 3 days upon SL-TBI.

B-D Histograms show numbers of cTEC (MHCII+UEA-1+), mTEC (MHCII+UEA-1+), TEC (MHCII+UEA-1+), mTEC (MHCII+UEA-1+) (B); TEC-enriched cells (defined as α6-integrin/Sca-1+ in the TEC subset) (C); and proliferating Ki-67+ cTECs, mTECs, and TEC-enriched cells (D).

E Expression of mRNAs coding for pro- (Bax, Bid, Bak) and anti-apoptotic (Bcl-xl) proteins analyzed by qPCR in purified cTECs and mTECs.

F Thymic sections from WT mice treated with GST or RANKL during 3 days upon SL-TBI were stained for the expression of K14 and Aire. The histogram shows the density of Aire+ cells in medullary area. m denotes the medulla. Fifteen sections were quantified for each condition; scale bar: 100 μm.

G, H The expression of Aire and TRA (Sp2 and Sp2) in purified mTECs (G) and Selp, Icam-1, Cc19, and Cc121 in purified cTECs (H) from WT mice treated with GST or RANKL was analyzed by qPCR.

Data information: Data are shown as mean ± SEM and are pooled from three independent experiments with similar results (n = 3–4 mice per group). *P < 0.05; **P < 0.01; ***P < 0.001, ****P < 0.0001. Exact P-values and statistical tests used to calculate them are provided in Appendix Table S2.
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RANKL improves thymic recovery

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Figure 3. RANKL administration induces LTA upregulation specifically in thymic LTI cells after TBI.

A
Expression of RANK receptor in thymic LTI cells from UT WT (n = 6) and at d3 SL-TBI (n = 6).
B
Expression level of LTA protein in thymic LTI cells from UT mice treated in vivo with PBS (n = 6), GST (n = 6), or RANKL-GST (n = 6) during 3 days after SL-TBI.
C
LTA protein was analyzed in thymocyte subsets and LTI cells from WT mice treated in vivo with GST (n = 9) or RANKL-GST (n = 9) during 3 days after SL-TBI.
D
Expression level of LTA protein in thymic LTI cells from WT mice treated in vivo with PBS (n = 6), an isotype control (Iso) (n = 3), or a neutralizing anti-RANKL antibody (IL225) (n = 6) during 3 days after SL-TBI.
E
LTA protein was analyzed in thymocyte subsets and LTI cells from WT mice treated in vivo with an isotype control (n = 3), or a neutralizing anti-RANKL antibody (n = 6) during 3 days after SL-TBI. Results are represented as fold change relative to the isotype condition.
F
Expression of LTA mRNA in the total thymus isolated from UT WT, RocR (−/−), and Zap70 (−/−) mice or at d3 SL-TBI (n = 3–6 mice per genotype).
G
Expression of LTA mRNA in the total thymus isolated from irradiated WT, Rag2 (−/−) mice, and WT mice treated 3 days before with dexamethasone (Dexa). Data are pooled of two to three experiments (n = 6–12 mice per group).
H
Expression level of LTA protein in thymic LTI cells during the course of BMT. pBMT: post-bone marrow transplantation. Data are pooled of three independent experiments with similar results (n = 3–4 mice per group).
I
Expression level of LTA protein in thymic LTI cells from UT WT mice or at d3, d6, d10, and d22 after BMT. Data are pooled of three independent experiments with similar results (n = 3–4 mice per group).
J
Expression level of LTA protein analyzed by flow cytometry in thymic LTI cells from CD45.1 donor and CD45.2 host origin at d3 and d6 after BMT. Data are pooled of four experiments (n = 3–4 mice per group).
K
RANKL protein expression in LTI cells from WT and LTA−/− mice at d3 SL-TBI. Data are pooled of three experiments (n = 3–5 mice per group).
L
The expression of LTA and LTAβ mRNA were measured by qPCR in the total thymus from UT WT mice (n = 4) or at d3 SL-TBI (n = 4).
M
MFI of LTA protein in CD45+ and CD45+ thymic cells from UT WT (n = 6) mice or at d3 SL-TBI (n = 6).
N, O
Representative histogram of LTA (N) and LTβR-Fc staining (O) in LTI cells from UT WT mice (n = 6) or at d3 SL-TBI (n = 6) or L-TBI (n = 6).

Data information: Data are shown as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Exact P-values and statistical tests used to calculate them are provided in Appendix Table S2.

as mTEC subsets in WT CD45.1:LTA−/− mice compared to WT CD45.1:WT controls at all time points analyzed (Fig 4B–D). Moreover, cTECs, mTECs, TECs, and TEPC-enriched cells were also reduced (Fig 4E and F). Importantly, total TECs, cTECs, mTECs, and TEPCs were less proliferative (Fig 4G). A reduced density of medullary Aire+ cells was still detectable at d65 after BMT (Appendix Fig S3), and consequently, the expression of Aire and its dependent TRAs (Sip1 and Sp2) were strongly affected (Fig 4H). The expression of an Aire-independent TRA (casein β) and Fez2 as well as its target genes (Apoa3, Fabp9, and Resp18) (Takaba et al., 2015) was also reduced. These data thus reveal that LTα is critical for TEC regeneration including TEPC-enriched cells during the course of BMT.

In line with these thymic environmental defects, thymocytes were reduced from DN to SP stage in WT CD45.1:LTA−/− mice after BMT (Fig 5A and B). Consequently, numbers of peripheral CD4 + and CD8 + T cells from CD45.1 donor origin were reduced in the blood and spleen of LTα−/− mice (Fig 5B), we analyzed early T-lineage progenitors (ETPs; CD4 + CD8− CD44+ CD25− Lin− CD117+). Whereas numbers of ETPs were normal in LTA−/− thymus at steady state, ETPs from CD45.1 donor origin were reduced in WT CD45.1: LTA−/− chimeras until 2 months after BMT (Fig 5C). This defect was not attributable to impaired hematopoietic progenitors because normal numbers of prethymic progenitors were observed in the BM of these mice (Fig 5C). We hypothesized that reduced ETPs could be due to a reduced homing capacity of circulating T-cell progenitors. Thymus homing is controlled by a multistep adhesion cascade initiated by P-selectin slowing down T-cell progenitors and allowing them to respond to CCL25, CCL21/19 gradients and to engage with ICAM-1 and VCAM-1 expressed by the thymic stroma, leading to a firm arrest (Rossi et al., 2005; Scimone et al., 2006; Krueger et al., 2010; Zlotoff et al., 2010). We found that purified TECs from WT CD45.1:LTA−/− mice showed a reduced expression of Ccl19 and Ccl21 mRNAs at d21 after BMT, whereas purified fibroblasts from these mice displayed a decrease expression of Ccl21 mRNA (Fig 5D). Furthermore, purified endothelial cells exhibited a reduced expression of Icam-1, Vcam-1, and Selp adhesion molecules (Fig 5D). TECs, fibroblasts and endothelial cells were thus defective in key molecules involved in thymus homing in BM-transplanted LTα−/− mice. To firmly demonstrate that thymus homing of T-cell progenitors was altered in LTA−/− mice, short-term homing assays were performed by injecting CD45.1 congenic BM cells into irradiated WT and LTA−/− recipients. LTA−/− thymus imported 3-fold less ETPs than WT thymus after thymic injury (Fig 5E). It is noteworthy that the effects of LTα on TEC and T-cell recovery were independent of those mediated by IL-23-regulated IL-22 described to be involved in thymic regeneration (Dudakov et al., 2012) since LTα−/− mice exhibited similar expression of these two cytokines after TBI (Fig 5F and G). Altogether, these results reveal that RANKL-regulated LTα constitutes an indispensable pathway for thymic regeneration.

RANKL administration enhances thymic regeneration upon BMT in an LTα-dependent manner

Since RANKL treatment improves TEC regeneration upon SL-TBI (Fig 2), we next evaluated whether RANKL boosts thymic recovery during the course of BMT. WT mice transplanted with CD45.1 BM cells were treated with RANKL-GST or GST at d2, d4, and d6 after BMT, and thymic regeneration was analyzed at d21 (Fig 6A) and d65 (Appendix Fig S4A) after BMT. In these experiments, as observed at d3 SL-TBI (Fig 3B), RANKL treatment also upregulated...
LTα in LTi cells, which was still detectable at d21 pBMT (Fig 6B). RANKL-treated WT CD45.1:WT mice showed increased medullary areas, numbers of TEC subsets, Aire+ mTEC frequency, and Aire-dependent TRAs compared to GST-treated mice (Fig 6C–F; Appendix Fig S4B). RANKL also increased numbers of CD31+ endothelial cells (Appendix Fig S5). Because we found that RANKL
Figure 4.
regulates LTα (Fig 3B), we next investigated whether these beneficial effects on TECs mediated by RANKL require LTα expression. The administration of RANKL in WT CD45.1:LTα−/− mice increased TEC numbers but to a lesser extent compared to RANKL-treated WT CD45.1:WT mice (Fig 6D; Appendix Fig S4B). In contrast, RANKL treatment in these mice did not enhance the size of the medulla, neither Aire+ mTEC frequency nor Aire-dependent TRAs, indicating that LTα is critical for the regeneration of Aire+ mTECs mediated by RANKL (Fig 6C, E and F).

Interestingly, RANKL treatment in WT CD45.1:WT chimeras substantially increased numbers of total donor cells and thymocytes of CD45.1 origin from ETP to SP stages at d21 and d65 upon BMT (Fig 6G; Appendix Fig S4C). In contrast, RANKL administration in WT CD45.1:LTα−/− mice had a poor effect on de novo thymopoiesis. To decipher the mode of action of RANKL on T-cell regeneration, we performed short-term homing assays in irradiated WT and LTα−/− mice treated with GST or RANKL. Strikingly, the receptivity capacity of circulating progenitors was substantially enhanced in RANKL-treated WT CD45.1:WT mice compared to GST-treated controls (Fig 6H). Importantly, this was not due to increased numbers of prethymic progenitors in the BM upon RANKL treatment (Fig 6H). In contrast, RANKL had no effect on ETP homing in WT CD45.1:LTα−/− mice. Consequently, RANKL treatment increased peripheral T-cell reconstitution only in WT CD45.1:WT mice after BMT (Fig EV4). Altogether, these data demonstrate that LTα is critical for optimal effects of RANKL administration on TEC regeneration, thymus homing of lymphoid progenitors, and T-cell reconstitution upon BMT.

Since the effects of RANKL-regulated LTα are independent of those mediated by IL-22 described to be involved in thymic regeneration (Fig 5F and G; Dudakov et al, 2012), we next investigated the respective efficiency of IL-22 and RANKL in thymic regeneration during BMT. To this, WT mice transplanted with CD45.1 BM cells were treated with IL-22, RANKL or both at d2, d4, and d6 after BMT and thymic regeneration was analyzed at d21. Whereas the concomitant administration of IL-22 and RANKL did not ameliorate thymic recovery compared to UT mice (Appendix Fig S6), we found that IL-22 and RANKL administered alone increased similarly numbers of developing T cells including ETPs and ameliorated peripheral T-cell reconstitution (Appendix Fig S6A–C). Interestingly, IL-22 alone increased preferentially numbers of mTECbl+ including CD80ahiAire+ and CD80ahi Aire+ mTEC subsets (Appendix Fig S6E and F), whereas RANKL alone enhanced numbers of all TEC populations including cTECs and mTECs (Appendix Fig S6D and E). The numbers of cTECbl, mTECbl, TECbl, mTECbo, and TEPC-enriched cells were also increased in RANKL-treated mice (Appendix Fig S6F and G). In addition to IL-22, RANKL thus constitutes a new therapy to enhance thymic regeneration upon BMT by ameliorating both TEC and T-cell reconstitution.

RANKL treatment also ameliorates thymic recovery upon BMT in aged individuals

Because the recovery of T-cell functions upon BMT is known to be delayed and less efficient in elderly patients compared to young individuals (Toubert et al, 2012), we finally investigated whether RANKL beneficial effects are persistent with age. To this, WT mice of 6–8 months, in which early thymic involution is characterized by a decline in TEC cellularity (Gray et al, 2006; Ki et al, 2014), were subjected to the same treatment described in Fig 6A. We found that RANKL increased numbers of cTECs, mTECs, and TEPC-enriched cells (Fig 7A and B). All thymocytes including ETPs were also increased in these mice (Fig 7C and D). Importantly, RANKL administration in WT CD45.1:LTα−/− chimeras did not improve significantly TEC cellularity compared to RANKL-treated WT CD45.1:WT mice (Fig 7A and B). Moreover, RANKL treatment had only minor effects on de novo thymopoiesis in WT CD45.1:LTα−/− chimeras (Fig 7C and D). Peripheral T-cell reconstitution was thus only enhanced in RANKL-treated WT CD45.1:WT mice (Appendix Fig S7). This set of data is consistent with the fact that LTi cells persisted with age and also upregulated LTα1β2 after TBI (Fig EV5A and B). Furthermore, BM-transplanted LTα−/− mice of 6–8 months of age showed defective TEC regeneration, de novo thymopoiesis, and peripheral T-cell reconstitution (Fig EV5C–K). Altogether, our data indicate that RANKL treatment boosts thymic recovery after BMT not only in young but also in older individuals in an LTα-dependent manner.

Discussion

Pre-BMT conditioning induces severe damages on the thymic microenvironment, which results in delayed lymphocyte production. It is therefore of paramount clinical interest to discover new molecules that enhance thymic regeneration for an efficient recovery of the immune system (van den Brink et al, 2004; Hollander et al, 2010).

Our study demonstrates that RANKL plays a crucial role in thymic recovery during BMT. Although that LTi cells strongly
Figure 5. LTα is required for de novo thymopoiesis during BMT.

A Numbers of total thymic cells and thymocyte subsets of CD45.1 donor origin were analyzed by flow cytometry in WT CD45.1:WT and WT CD45.1:LTα<sup>−/−</sup> mice at d10, d21, and d65 after BMT.

B Numbers of DN1 (CD4<sup>+</sup>CD25<sup>−</sup>), DN2 (CD4<sup>+</sup>CD25<sup>+</sup>), DN3 (CD4<sup>+</sup>CD25<sup>−</sup>), and DN4 (CD4<sup>+</sup>CD25<sup>−</sup>) of CD45.1 origin were also analyzed at d10 and d21 after BMT.

C Numbers of early thymic progenitors (ETPs; CD4<sup>+</sup>CD8<sup>−</sup>CD117<sup>+</sup>) and BM pre-thymic progenitors (CD3<sup>+</sup>CD4<sup>+</sup>Lin<sup>+</sup>CD117<sup>+</sup>) were analyzed by flow cytometry in UT WT and LTα<sup>−/−</sup>/C0 mice or WT CD45.1:WT and WT CD45.1:LTα<sup>−/−</sup>/C0 chimeras at d65 after BMT.

D The expression of Ccl19, Ccl21, Ccl25, Icam-1, Vcam-1, and Selp mRNAs was measured by qPCR in purified EpCAM<sup>+</sup> TECs, CD31<sup>+</sup>EpCAM<sup>+</sup> endothelial cells, and gp38<sup>+</sup>EpCAM<sup>+</sup> fibroblasts isolated from WT CD45.1:WT (n = 5) and WT CD45.1:LTα<sup>−/−</sup> (n = 5) chimeras at d21 after BMT.

E Numbers of ETPs and total cells of CD45.1 donor origin in CD45.2 WT or LTα<sup>−/−</sup> recipients 48 h after i.v. injection of CD45.1 BM cells.

F Expression of Il-22 mRNA in the total thymus isolated from UT WT and LTα<sup>−/−</sup> mice (n = 4) or at d3 SL-TBI (n = 4).

G Expression of Il-23 mRNA in the total thymus and purified DCs isolated from UT WT and LTα<sup>−/−</sup> mice (n = 4) or at d3 SL-TBI (n = 4).

Data information: Data are shown as mean ± SEM and are pooled of three independent experiments with similar results (n = 3–5 mice per group). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Exact P-values and statistical tests used to calculate them are provided in Appendix Table S2.
Figure 6.
upregulated RANKL after irradiation, radio-resistant CD4+ SP cells that expressed lower levels of RANKL constitute the major source of RANKL after TBI, which is in line with their high numbers after SL-TBI. Based on the use of a neutralizing anti-RANKL antibody and ex vivo RANKL administration, our data reveal that this cytokine induces LTα upregulation specifically in LTi cells after irradiation. We thus propose a model in which the effects on thymic regeneration of RANKL-regulated LTα expressed by LTi cells are strengthened by CD4+ thymocytes via RANKL upregulation after irradiation (Appendix Fig S8). Since RANKL, LTα, and RORγt transcription factor were upregulated in thymic LTi cells after TBI, we believe that these cells change their phenotype upon stress-induced thymic damage. Thymic LTi cells are thus likely in a “quiescent stage” at steady state and are activated after irradiation to repair the injured tissue. When considering that LTi cells expressing RANKL and LTα are involved in the organogenesis of lymph nodes (Yoshida

Figure 6. RANKL boosts TEC regeneration and de novo thymopoiesis in an LTα-dependent manner upon BMT.

A Experimental setup: WT CD45.1-WT and WT CD45.1-LTα−/− chimeras were treated with GST or RANKL-GST proteins at d2, d4, and d6 after BMT and TEC regeneration and T-cell reconstitution were analyzed at d21 after BMT.
B Expression level of LTα protein in thymic LTi cells in UT mice or treated with GST or RANKL-GST.
C Thymic sections from WT CD45.1-WT and WT CD45.1-LTα−/− mice treated with GST and RANKL at d2, d4, and d6 after BMT were stained for the expression of K14 at d21 pBMT. The histogram shows quantifications of medullary areas. m and c denote the medulla and the cortex, respectively. Twenty sections were quantified for each condition; scale bar: 100 μm.
D, E Numbers of total TECs, cTECs, and mTECs (D) and flow cytometry profiles of Aire+ mTECs in total EpCAM+ TECs (E).
F Expression of mRNAs coding for TRAs (Sp1 and Sp2) in CD45-thymic stromal cells analyzed by qPCR.
G Numbers of total cells and thymocyte subsets of CD45.1 donor origin analyzed in the thymus.
H Numbers of ETPs of CD45.1 donor origin in the BM from CD45.2 WT or LTα−/− recipients 48 h after i.v. injection of CD45.1 BM cells.

Data information: Data are shown as mean ± SEM and are pooled of three independent experiments with similar results (n = 3–5 mice per group). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Student’s t-test. Exact P-values are provided in Appendix Table S2.

Figure 7. Beneficial effects mediated by RANKL treatment on thymic regeneration after BMT require LTα expression in aged mice.

A, B Numbers of total TECs, cTECs, mTECs (A), and TEPC-enriched cells (B) were analyzed at d21 upon BMT in the thymus from WT CD45.1-WT and WT CD45.1-LTα−/− chimeras of 6–8 months of age treated with GST or RANKL proteins.
C, D Numbers of total thymic cells, thymocyte subsets (C), and ETPs (D) of CD45.1 origin.

Data information: Data are shown as mean ± SEM and are pooled of two independent experiments with similar results (n = 3 mice per group). *P < 0.05; one-tailed Mann–Whitney U-test. Exact P-values are provided in Appendix Table S2.
et al., 1999), our data suggest that thymic LTi cells likely reactivate an embryonic program to repair the thymus after irradiation.

Interestingly, we found that the administration of a neutralizing anti-RANKL antibody led to an impaired TEC regeneration, highlighting the importance of RANKL in endogenous TEC recovery. Conversely, *ex vivo* RANKL administration after TBI boosted the regeneration of TEC subsets including TEPc-enriched cells. Furthermore, flow cytometry, histology, and qPCR experiments indicated that RANKL also boosted the regeneration of Aire+ mTECs. Importantly, we demonstrate that RANKL treatment during the early phase of BMT enhanced numbers of TECs and thymocytes including ETPs as well as thymus homing of T-cell progenitors and T-cell output.

Notably, although mice were treated during the early phase of BMT, RANKL treatment had long-term beneficial effects detectable until 2–3 months after BMT on both TEC and T-cell compartments. Improved T-cell reconstitution can be explained by increased stromal niches linked to increased TEC cellularity but also to enhanced thymus homing of lymphoid progenitors, which is a critical step for ameliorating T-cell recovery (Penit & Ezine, 1989; Chen et al., 2004; Zlotoff et al., 2011). The latter effect is likely mediated by increased expression of adhesion molecules and chemokines involved in this process. Nevertheless, we cannot exclude that the enhanced thymus homing in RANKL-treated mice is also favored by increased vasculature, which is important for T-cell progenitor colonization (Lind et al., 2001). Furthermore, given that RANKL administration upon BMT increased primitive progenitors in the BM, which correlated with the development of active osteoclasts (Appendix Fig S9; Kollet et al., 2006), we thus cannot also exclude that *de novo* thymopoiesis is also favored by increased hematopoietic progenitors. Whereas RANKL induces mTEC differentiation in the steady-state thymus (Rossi et al., 2007; Akiyama et al., 2008; Hikosaka et al., 2008; Ohigashi et al., 2011; Roberts et al., 2012), our data reveal that RANKL plays distinct roles in thymic regeneration during BMT.

We further found that RANKL induced LTα upregulation specifically in LTi cells, which express its cognate receptor, RANK. In contrast, LTα did not regulate RANKL, indicating that LTα acts downstream of RANKL. Whereas LTα is dispensable at steady state for TEC and T-cell cellularity, we found that LTα is critical for the recovery of thymic function. TEC subsets including TEPc-enriched cells were severely reduced in LTα−/−-transplanted recipients from up to 2 months after BMT. Moreover, all thymocyte subsets as well as ETPs were also reduced in these mice likely due to defective thymus homing capacity. Consistently, we found that LTα expression is required for the expression of adhesion molecules and chemokines, known to promote T-cell progenitor entry in the thymus, in stromal cells during BMT. In accordance with our data, it has been recently reported that LTβR regulates at steady state thymus homing by controlling VCAM-1 and ICAM-1 on endothelial cells (Lucas et al., 2016; Shi et al., 2016). Peripheral T-cell reconstitution is thus altered in BM-transplanted LTα−/− mice, which is explained by a diminished thymic export of CD4+ and CD8+ T cells revealed by a weak frequency of recent thymic emigrants. Whereas LTα is involved in the regular thymic architecture at steady state (Boehm et al., 2003; Seach et al., 2008; Irla et al., 2012, 2013), our data therefore reveal a new function for LTα in controlling TEC and T-cell regeneration during BMT.

It has been described that the IL-10 family cytokine, IL-22, participates to thymus recovery (Dudakov et al., 2012). Our results show that RANKL-regulated LTα represents a distinct pathway of that mediated by IL-22 in thymic regeneration. Furthermore, since IL-22 and LTα are both provided by LTi cells, it is likely that this cell type uses different mechanisms for thymic repair. Surprisingly, the concomitant administration of IL-22 and RANKL cytokines did not show any benefits on thymic recovery likely due to a too strong signal that results in an ineffective action on thymic recovery. Alternatively, this could be explained by a neutralization of the cell signals mediated by these two cytokines. However, while IL-22 and RANKL administered alone show similar benefits on T-cell reconstitution, they do not exhibit the same effects on TEC regeneration with a preferential effect for IL-22 on mTEC and a more global effect for RANKL on all TEC subsets. These data thus indicate that IL-22 and RANKL play distinct roles in TEC recovery. Furthermore, one would expect that thymic regeneration during the course of BMT is also defective in RANKL−/− mice. Unfortunately, we were unable to test this hypothesis since RANKL−/− mice show severe growth retardations and exhibit a drastic reduction in mTECs (Kong et al., 1999; Hikosaka et al., 2008). Nevertheless, we demonstrate that *in vivo* RANKL neutralization impairs TEC regeneration, whereas RANKL treatment has beneficial effects on thymic recovery. Moreover, RANKL administration in BM-transplanted LTα−/− mice had only minor effects on TEC regeneration, numbers of ETPs and strikingly *de novo* thymopoiesis was not ameliorated. Interestingly, whereas LTα is dispensable for Aire+ mTEC differentiation at steady state (Venanzi et al., 2007), the regeneration of these cells induced by RANKL treatment critically depends on LTα. These data indicate that the mechanisms involved in Aire+ mTEC regeneration are thus distinguishable from those implicated in their emergence/differentiation at steady state. Importantly, beneficial effects of RANKL treatment in TEC regeneration are likely not due to a direct action on TECs because the effects of RANKL on these cells were modest in absence of LTα. Thus, these data argue in favor of model in which RANKL acts indirectly on TECs through LTα overexpression in LTi cells. This notion is supported by the fact that the turnover rate of mTECs is of around 2 weeks (Gabler et al., 2007; Gray et al., 2007), and thus, it is unlikely that RANKL injected early after BMT still acts on mTECs 2 months later.

Interestingly, RANKL administration is also efficient for TEC and T-cell regeneration in older individuals in which thymic involution results in diminished TEC cellularity, disrupted thymic architecture and decreased T-cell output (Gray et al., 2006; Chinn et al., 2012). These results are of special interest for elderly patients in which the recovery of T-cell functions upon BMT is less efficient (Toubert et al., 2012). This study thus reveals that the administration of RANKL offers an innovative clinical strategy to boost thymic recovery in young and aged individuals at several levels: TEC regeneration, thymus homing of T-cell progenitors, and *de novo* thymopoiesis. To avoid any potential side effects of the systemic administration of RANKL, such as osteoporosis, possible strategies would be to deliver directly this molecule intrathymically in patients after cytotoxic conditioning or to combine RANKL with biphosphonates to prevent bone resorption (Tomimori et al., 2009). The administration of biphosphonates induces apoptosis of mature osteoclasts (Hughes et al., 1995; Drake et al., 2008) and importantly does not affect RANKL synthesis (Kim et al., 2002; Verde et al., 2015). In conclusion, RANKL in clinic is expected to be promising for enhancing the regeneration of immune functions in patients whose thymus has been severely damaged.
Materials and Methods

**Mice**

CD45.1 and CD45.2 WT (Janvier), CD45.2 LTα−/− (De Togni et al, 1994), Rorc<sup>Ctpp/Ctpp</sup> knock-in (Eberl et al, 2004), ZAP-70<sup>−/−</sup> (Wiest et al, 1997), and Rag2<sup>−/−</sup> (Shinkai et al, 1992) mice were on B6 background and maintained under specific pathogen-free conditions at the CIML, AniCan (Lyon, France) or IGMM (Montpellier, France). Standard food and water were given ad libitum. Males and females were used at the age of 6–8 weeks for each mouse strain. Chimeras were generated at 6–8 weeks or 6–8 months of age.

**Thymic damage and BM chimeras**

Total body irradiation was performed with a Cs-137 γ-radiation source. Sublethal-TBI (SL-TBI) was performed with 500 rads with the Foxp3 staining kit (eBioscience). Foxp3 and Ki-67 intracellular stainings were performed with the Foxp3 staining kit (eBioscience). Aire, LTα, RANK, and RORγt intracellular stainings were performed with BD Cytofix/Cytoperm and Perm/Wash buffers. For staining with LTβR-Fc, cells were incubated with LTβR-Fc (RnD systems) at 1 μg/10<sup>6</sup> cells for 45 min on ice. LTβR-Fc staining was visualized using an Alexa Fluor 488-conjugated donkey anti-human IgG F(ab’)<sub>2</sub> fragment (1:400; Jackson ImmunoResearch). Flow cytometry analysis was performed with a FACSCanto II (BD), and data were analyzed with FlowJo software.

**Quantitative RT–PCR**

Total RNA was prepared with TRIzol (Invitrogen). cDNAs were synthesized with oligo(dT) using Superscript II reverse transcriptase (Invitrogen). qPCR was performed with the ABI 7500 fast real-time PCR system (Applied Biosystem) and SYBR Premix Ex Taq master ware using unpaired Student’s t-test, Mann–Whitney test, or ANOVA on multiple variable analyses *p < 0.05; **p < 0.01.

**Statistical analysis**

Statistical significance was assessed with GraphPad Prism 6 software using unpaired Student’s t-test, Mann–Whitney test, or ANOVA on multiple variable analyses *p < 0.05; **p < 0.01;
The paper explained

Problem
Cytoablative treatments commonly used to prepare patients to bone marrow transplantation (BMT) severely affect thymic stromal cells, which results in delayed T-cell reconstitution. This prolonged deficiency in T cells could result in severe clinical consequences characterized by an increased susceptibility to opportunistic infections, autoimmunity, tumor relapse, or the development of secondary malignancies and thus could lead to post-transplant morbidity and mortality. Although the mechanisms that govern thymic regeneration are crucial for the recovery of a functional immune system, they remain poorly understood. Furthermore, the identification of therapeutic molecules that boost thymic regeneration is of clinical interest to efficiently improve T-cell reconstitution and thus to prevent a period of compromised immunity after cytotoxic treatments.

Results
Here, we found that RANKL, a TNF family member, is naturally upregulated in CD4+ thymocytes and lymphoid tissue inducer (LTi) cells during the early phase of thymic recovery. Importantly, the in vivo neutralization of RANKL impairs thymic epithelial cell (TEC) regeneration, whereas the ex vivo administration of RANKL protein boosts thymic recovery during the course of BMT. We further demonstrate that RANKL treatment after total body irradiation induces specifically in LTi cells, LTi expression, which is critical for the regeneration of TECs and de novo thymopoiesis. Importantly, thymic recovery improved by RANKL administration strictly depends on LTi expression. Furthermore, RANKL treatment boosts thymic recovery upon BMT not only in young but also in aged individuals.

Impact
In the present work, we identified RANKL as a new potential candidate for boosting thymic regeneration after thymic damage. This finding opens a new perspective for ameliorating the recovery of T-cell functions not only after cytotoxic therapy but also in patients with complications related to aging process, HIV/AIDS, malnutrition, or radiation poisoning due to nuclear disaster.

***p < 0.001, ****p < 0.0001. Normal distribution of the data was assessed using d’Agostino-Pearson omnibus normality test. Correlations were calculated using the nonparametric Spearman correlation test. Error bars represent mean ± SEM. Exact P-value and statistical test used for each figure are provided in Appendix Table S2.

Study approval
Experiments were performed in accordance with the animal care guidelines of the European Union and French laws. All animal procedures were approved by and performed in accordance with guidelines of the Centre d’Immunologie de Marseille-Luminy (CIML) and AniCan (Lyon, France).

Expanded View for this article is available online.

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Author contributions
NL, HV, JM, and MI performed experiments and analyzed the data. NL and MI interpreted data and wrote the manuscript. MI supervised and conceived the study.

Conflict of interest
The authors declare that they have no conflict of interest.

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