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Research Article

IL-7 derived from lymph node fibroblastic reticular cells is dispensable for naive T cell homeostasis but crucial for central memory T cell survival

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The survival of peripheral T cells is dependent on their access to peripheral LNs (pLNs) and stimulation by IL-7. In pLNs fibroblastic reticular cells (FRCs) and lymphatic endothelial cells (LECs) produce IL-7 suggesting their contribution to the IL-7-dependent survival of T cells. However, IL-7 production is detectable in multiple organs and is not restricted to pLNs. This raises the question whether pLN-derived IL-7 is required for the maintenance of peripheral T cell homeostasis. Here, we show that numbers of naive T cells (TN) remain unaffected in pLNs and spleen of mice lacking IL7 gene activity in pLN FRCs, LECs, or both. In contrast, frequencies of central memory T cells (TCM) are reduced in FRC-specific IL-7 KO mice. Thus, steady state IL-7 production by pLN FRCs is critical for the maintenance of TCM, but not TN, indicating that both T cell subsets colonize different ecological niches in vivo.

Keywords: central memory T cells · fibroblastic reticular cells · IL-7 · naive T cells · T cell homeostasis

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

IL-7 is indispensable for naive (TN) and memory T cell (TM) survival [1,2]. Correspondingly, IL-7-deficient (IL-7−/−) mice suffer from severe lymphopenia [3] and adoptively transferred TN fail to survive in such recipients [4]. Conversely, the administration of recombinant IL-7 supports T cell survival, e.g., via the upregulation of anti-apoptotic B-cell lymphoma-2 (Bcl-2) [1], myeloid cell leukemia-1 (Mcl-1) [5], and the promotion of metabolic functions [6–8].

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The maintenance of the T<sub>N</sub> pool relies on the accessibility of secondary lymphoid organs (SLOs) where IL-7 is produced by lymphoid stromal cells (LSCs) [9]. In peripheral lymph nodes, for example, fibroblastic reticular cells (FRCs) and lymphatic endothelial cells (LECs) are the main sources of IL-7 [9]. Co-culture experiments demonstrated that FRC-derived IL-7 promotes T cell survival [9]. IL-7 binds to the ECM [10,11] suggesting that it might exert its function in close vicinity to the site of production. Due to the facts that T cell survival is impaired in vivo if either IL-7 action or peripheral LN (pLN) access is blocked [1,4,9,12,13], it has been proposed that circulating T<sub>N</sub> receive IL-7-dependent survival signals in pLNs [14–17]. Since T<sub>CM</sub> and T<sub>N</sub> have similar migration patterns in vivo [18] and both rely on IL-7 [1,4], pLN FRCs are supposed to be critical for the IL-7-dependent persistence of both T cell subsets in vivo [15,17]. A potential contribution of LEC-derived IL-7 has been suggested as well [19].

However, various non-hematopoietic stromal cells express IL-7 [20–23] and its steady state levels vary strongly between different organs [24,25]. For example, intestine and skin produce high levels of IL-7 in the steady state while only low levels of IL-7 gene activity are detectable in the adult liver [24,25]. Since T<sub>N</sub> and T<sub>CM</sub> continuously recirculate between SLOs, blood, and lymph [26], they might utilize IL-7 derived from various organs. Hence, it remained unclear whether the maintenance of peripheral T cell homeostasis relies on the local action of IL-7 in pLNs and/or systemic effects of IL-7 produced by alternative sources.

In order to answer this question, we generated conditional IL-7<sup>fl/fl</sup> KO (IL-7<sup>fl/fl</sup>) mice and inactivated IL-7<sup>fl/fl</sup> gene activity in a cell type-specific manner in pLNs. Here, we show that T<sub>N</sub> numbers remained unaltered in pLNs and spleens of LEC- and FRC-specific IL-7<sup>fl/fl</sup> KO (LEC<sup>ΔIL-7</sup> and FRC<sup>ΔIL-7</sup>) mice. In apparent contrast, T<sub>CM</sub> abundance was significantly reduced in FRC<sup>ΔIL-7</sup> mice, an effect that was most pronounced for CD8<sup>+</sup> T<sub>CM</sub> in pLNs. In summary, we provide evidence that FRC-derived IL-7 is dispensable for the systemic survival of T<sub>N</sub> cells. On the contrary, however, IL-7 produced by pLN FRCs is crucial for the maintenance of T<sub>CM</sub> homeostasis indicating that T<sub>N</sub> and T<sub>CM</sub> occupy different ecological niches in vivo.

Results

Ubiquitous IL-7 gene inactivation impairs peripheral T cell homeostasis

In order to elucidate whether pLN-derived IL-7 is crucial for the maintenance of peripheral T cell homeostasis, we generated conditional IL-7 KO (IL-7<sup>−/−</sup>) mice (Supporting Information Fig. 1A). IL-7<sup>−/−</sup> mice were crossed to conventional IL-7<sup>−/−</sup> KO (IL-7<sup>−/−</sup>) mice [3] and mice ubiquitously expressing the loxP-specific recombinase Cre (PGK-Cre<sup>+</sup>) [27] to obtain PGK-Cre<sup>+</sup> IL-7<sup>−/−</sup> mice. PGK-Cre-mediated inactivation of the IL-7<sup>fl</sup> allele was very efficient as shown by the fact that IL-7 mRNA was undetectable in PGK-Cre<sup>+</sup> IL-7<sup>−/−</sup> mice (Supporting Information Fig. 1B).

Next, we compared the impact of conditional and conventional IL-7 gene inactivation on IL-7-dependent lymphocyte homeostasis. While mice harboring one intact IL-7<sup>wt</sup> allele (PGK-Cre<sup>−</sup> IL-7<sup>−/−</sup>, PGK-Cre<sup>−</sup> IL-7<sup>−/−</sup>, and PGK-Cre<sup>−</sup> IL-7<sup>−/−</sup>) mice) had comparable numbers of T and B cells in the spleen, ubiquitous IL-7 gene inactivation in PGK-Cre<sup>−</sup> IL-7<sup>−/−</sup> mice was associated with a strong decrease of T and B cell numbers similar to IL-7<sup>−/−</sup> mice (Fig. 1A). Importantly, the lack of IL-7 production in PGK-Cre<sup>−</sup> IL-7<sup>−/−</sup> and IL-7<sup>ΔIL-7</sup> mice was accompanied by the selective reduction of CD4<sup>+</sup>CD62L<sup>lo</sup> CD4<sup>+</sup> and CD8<sup>+</sup> T<sub>N</sub> as well as the enrichment of CD4<sup>+</sup> CD4<sup>+</sup> and CD8<sup>+</sup> memory T cells (T<sub>M</sub>; Fig. 1B and C).

In summary, IL-7-dependent T cell homeostasis is similarly impaired in IL-7<sup>−/−</sup> and PGK-Cre<sup>−</sup> IL-7<sup>−/−</sup> mice thus confirming (i) the efficient Cre-mediated inactivation of the IL-7<sup>fl</sup> allele and (ii) the crucial importance of IL-7 for T<sub>N</sub> generation and maintenance. Hence, our IL-7<sup>−/−</sup> mouse is a suitable tool to study the impact of pLN-specific IL-7 gene inactivation on peripheral T cell homeostasis.

LEC-derived IL-7 is dispensable for peripheral T cell homeostasis

In pLNs, CD45<sup>−</sup> stromal cells comprise gp38<sup>+</sup>CD31<sup>+</sup> FRCs, gp38<sup>+</sup>CD31<sup>−</sup> LSCs, gp38<sup>+</sup>CD31<sup>−</sup> blood endothelial cells (BECs) and gp38<sup>+</sup>CD31<sup>−</sup> double negative cells (DNs) [28] (Fig. 2A). Lyve1-expressing LECs produce IL-7 in pLNs and throughout the body [29] and are supposed to be important regulators of IL-7-dependent peripheral T cell homeostasis [19]. In order to test this hypothesis, we generated LEC<sup>ΔIL-7</sup> mice lacking IL-7 gene expression specifically in LECs. For this purpose, Lyve1-Cre-transgenic (Lyve1-Cre<sup>+</sup>) mice [30] were crossed to IL-7<sup>−/−</sup> mice. Lyve1-Cre<sup>+</sup> mice harboring at least one intact IL-7 allele (LEC<sup>wt</sup> mice) served as controls. CD4<sup>+</sup> stromal cells were purified from LNs of LEC<sup>ΔIL-7</sup> and LEC<sup>wt</sup> mice and relative IL-7 mRNA levels were quantified by RT-qPCR. In agreement with a previous report [9], LECs produced considerable amounts of IL-7 mRNA in control mice, even though tenfold less than FRCs (Fig. 2A). Of note, IL-7 mRNA levels were strongly reduced in LECs from LEC<sup>ΔIL-7</sup> mice indicating successful IL-7 gene inactivation. On the contrary, IL-7 mRNA levels in FRCs, BECs, and DNs were comparable in LEC<sup>ΔIL-7</sup> and LEC<sup>wt</sup> mice.

In order to study whether LEC-derived IL-7 affects peripheral T cell homeostasis, CD4<sup>+</sup> and CD8<sup>+</sup> T cells were quantified in pLNs and spleens of LEC<sup>ΔIL-7</sup> and LEC<sup>wt</sup> mice. As shown in Fig. 2B, T cell numbers were indistinguishable between both mouse lines. Furthermore, relative frequencies and numbers of CD4<sup>+</sup>CD62L<sup>lo</sup> T<sub>N</sub>, CD4<sup>+</sup>CD62L<sup>lo</sup> T<sub>Em</sub>, and CD4<sup>+</sup>CD62L<sup>lo</sup> T<sub>CM</sub> were comparable in pLNs and spleens (Fig. 2C–H), although CD4<sup>+</sup> T<sub>CM</sub> frequencies were reduced in pLNs of LEC<sup>ΔIL-7</sup> mice (Fig. 2C and D). In conclusion, IL-7 gene inactivation in LECs does not have major effects on quantitative and qualitative aspects of peripheral T cell homeostasis.
Figure 1. Ubiquitous Il7 gene inactivation impairs T cell homeostasis. (A) Absolute cell numbers of CD3^+CD4^+ or CD3^+CD8^+ T cells and B220^+ B cells were determined in the spleen of the indicated mouse lines. (B and C) Shown are representative contour plots for the CD44/CD62L expression profiles of (B) CD3^+CD4^+ or (C) CD3^+CD8^+ T cells in spleen. Numbers in contour plots indicate percentages. Frequencies of naive (B) CD3^+CD4^+CD44LOCD62LHI and (C) CD3^+CD8^+CD44LOCD62LHI T cells are summarized in bar diagrams. (A–C) The data displayed in bar diagrams represent mean ± SEM of seven to nine mice per group analyzed in two independent experiments by flow cytometry. Statistical significances were tested using a non-parametric two-tailed Mann–Whitney U-test (*p < 0.05; **p < 0.01; ***p < 0.001).

FRC-derived IL-7 does not affect size and TCR diversity of the peripheral T cell pool

Prx1-Cre-transgenic (Prx1-Cre^+) mice express Cre in BM stromal cells [31], which are crucial for IL-7-dependent B cell development [32]. Whether this mouse model is suitable for targeting FRCs in pLNs was analyzed next. For this purpose, Prx1-Cre^+ mice were crossed to ROSA26 reporter mice expressing red fluorescent protein (RFP) upon Cre-mediated activation of the reporter construct [33]. Peripheral LNs of Prx1-Cre^+ ROSA26RFP mice were analyzed by flow cytometry to determine the degree of cell type-specific recombination. Among CD45^- stromal cells, around 80% of FRCs expressed RFP while LECs, BECs, and DNs showed only negligible levels of recombination (Fig. 3A and B). Of note, Cre activity was barely detectable in CD45^+ immune cells (Fig. 3A) as well as splenic LSCs (data not shown). Hence, Prx1-Cre^+ mice allow gene targeting in pLN FRCs.

In order to inactivate Il7 gene activity in pLN FRCs, Prx1-Cre^+ mice were crossed to IL-7fl/fl mice. As compared to FRCwt littermate controls, Il7 mRNA levels were reduced by approximately 83% in pLNs of FRC^IL-7 mice (Fig. 3C) confirming that FRCs are the major source of IL-7 in pLNs. In contrast, Il7 mRNA levels in the spleen of FRC^IL-7 mice remained unaltered (Fig. 3C), probably due to the different developmental origins of splenic and LN FRCs [34,35]. Importantly, FRC-specific Il7 inactivation did not affect frequencies of LSC subsets (Fig. 3D), overall morphology, and chemokine secretion in pLNs (Supporting Information Fig. 2A–D).

When CD4^+ and CD8^+ T cells were quantified in pLNs and spleens of FRC^IL-7 and FRCwt mice, no significant differences
LEC-derived IL-7 is dispensable for peripheral T cell homeostasis. (A) Based on their differential expression of gp38 and CD31, live TER-119\(^-\)CD45\(^-\)pLN LSCs can be subdivided into gp38\(^+\)CD31\(^-\) FRCs, gp38\(^+\)CD31\(^+\) LECs, gp38\(^-\)CD31\(^+\) BECs, and gp38\(^-\)CD31\(^-\) DNs. Shown is a representative contour plot from LEC\(^/\Delta I L-7\) mice; numbers indicate percentages. The indicated LSC subsets were purified by flow cytometry from LNs of LEC\(^wt\) (Lyve1-Cre\(^+\)IL-7\(^-\)/wt and Lyve1-Cre\(^-\)IL-7wt/wt) and LEC\(^/\Delta I L-7\) (Lyve1-Cre\(^+\)IL-7\(^-\)/fl ) mice. Three independent sorts with pooled pLNs from three to four mice per group (in total nine to ten mice per group) were performed. Relative \(I l 7\) mRNA amounts were determined by RT-qPCR in relation to \(H p r t\). Data displayed in the bar diagram are representative of two data points per group analyzed in two independent RT-qPCR experiments and show mean \(\pm\) SEM of triplicates. Statistical significances were tested using a nonparametric two-tailed Mann–Whitney \(U\)-test (\(* p < 0.05\)). (B) Absolute numbers of CD3\(^+\)CD4\(^+\) and CD3\(^+\)CD8\(^+\) T cells were determined in pLNs and spleen (Sp). (C, D, F, and G) Frequencies and (E and H) absolute numbers of naive (TN; CD4\(^lo\)CD62L\(^hi\)), effector memory (TEM; CD4\(^hi\)CD62L\(^lo\)) and central memory (TCM; CD44\(^hi\)CD62L\(^hi\)) T cells were determined after gating on (C–E) CD3\(^+\)CD4\(^+\) and (F–H) CD3\(^+\)CD8\(^+\) cells isolated from pLNs or spleen. (C and F) Shown are representative contour plots and numbers indicate percentages. (B–H) Data was collected using flow cytometry. (B–H) The data shown in bar diagrams represent mean \(\pm\) SEM combined from 11–12 LEC\(^wt\) (Lyve1-Cre\(^+\)IL-7\(^-\)/wt) and LEC\(^/\Delta I L-7\) (Lyve1-Cre\(^+\)IL-7\(^-\)/fl ) mice per group analyzed in six independent experiments. Statistical significances were tested using a non-parametric two-tailed Mann–Whitney \(U\)-test (* \(p < 0.05\)).

were detected in either case (Fig. 3E and G). Furthermore, TCR V\(\beta\) repertoires of CD4\(^+\) and CD8\(^+\) T cells were indistinguishable between FRC\(^/\Delta I L-7\) and FRC\(^wt\) mice (Fig. 3F and H). Hence, the size and diversity of the peripheral T cell pool is independent of pLN FRC-derived IL-7.

Next, we assessed whether LEC-derived IL-7 compensates for the lack of FRC-derived IL-7. For this purpose, FRC\(^/\Delta I L-7\) mice were crossed to LEC\(^/\Delta I L-7\) mice to generate double Cre-transgenic FRC/LEC\(^/\Delta I L-7\) mice lacking \(I l 7\) gene expression in both, FRCs and LECs. Similar to LEC\(^/\Delta I L-7\) (Fig. 2B) or FRC\(^/\Delta I L-7\) mice (Fig. 3E and G), CD4\(^+\) and CD8\(^+\) T cells were equally abundant in FRC/LEC\(^/\Delta I L-7\) and FRC/LEC\(^wt\) controls (Fig. 3I and J) arguing against a compensatory effect of LEC-derived IL-7 in FRC\(^/\Delta I L-7\) mice.
Figure 3. FRC-derived IL-7 does not affect size and diversity of the peripheral T cell pool. (A and B) Peripheral LNs were isolated from Prx1-Cre^+^ ROSA26^RFP^ (white curves) and Prx1-Cre^−^ ROSA26^RFP^ (grey curves) mice to determine recombination efficiency in live TER-119^−^ CD45^−^ LSCs and TER-119^−^ CD45^+^ leukocytes. (A) Numbers indicate percentages of RFP^+^ cells in Prx1-Cre^+^ ROSA26^RFP^ mice. (B) Data show percentages of RFP^+^ cells for the indicated LSC subsets (mean ± SEM). Data were pooled from four to five independent experiments with one to three mice per group. For FRCs, LECs, BECs, and DNs 13, 8, 13, and 13 individual data points were acquired, respectively. (C) Relative Il7 mRNA amounts were determined.

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FRC-derived IL-7 determines T<sub>CM</sub> abundance

Bcl-2 is a direct target of IL-7 [1] and is expressed at particularly high levels by CD8<sup>+</sup> T<sub>TM</sub> [36]. In order to test whether Bcl-2 expression is altered in the absence of FRC-derived IL-7, CD8<sup>+</sup> T<sub>N</sub> and T<sub>CM</sub> derived from pLN and spleens were analyzed. As shown in Fig. 4A, frequencies and numbers of CD4<sup>+</sup>Bcl-2<sup>+</sup> CD8<sup>+</sup> T<sub>TM</sub> were significantly reduced in pLN, but not spleens, of FRC<sup>−/−</sup> mice. IL-7 conditions CD8<sup>+</sup> T cell responses for the IL-15-induced upregulation of Eomesodermin (Eomes) [37], a transcription factor promoting TM differentiation [38]. As shown in Fig. 4B, CD4<sup>+</sup>Eomes<sup>hi</sup> CD8<sup>+</sup> T<sub>TM</sub> were strongly reduced in pLN of FRC<sup>−/−</sup> mice. Again, these differences between FRC<sup>−/−</sup> and FRC<sup>+/+</sup> mice were most evident in pLN. However, there was a tendency of reduced CD8<sup>+</sup> T<sub>CM</sub> frequencies and cell numbers in spleens of FRC<sup>−/−</sup> mice (Fig. 4A and B).

To analyze this IL-7-dependent T<sub>M</sub> defect in more detail, CD4<sup>+</sup> and CD62L<sup>+</sup> expression was analyzed on CD8<sup>+</sup> T cells from pLN and spleens of FRC<sup>−/−</sup> mice. Frequencies and numbers of CD8<sup>+</sup> CD4<sup>+</sup>CD62L<sup>hi</sup> T<sub>N</sub> and CD4<sup>+</sup>CD62L<sup>lo</sup> T<sub>CM</sub> were indistinguishable in pLN and spleens of FRC<sup>+/+</sup> and FRC<sup>−/−</sup> mice (Fig. 4C–E). In apparent contrast, frequencies of CD8<sup>+</sup> CD4<sup>+</sup>CD62L<sup>hi</sup> T<sub>CM</sub> were significantly reduced in pLN and spleens of FRC<sup>−/−</sup> mice (Fig. 4D). With regard to absolute CD8<sup>+</sup> T<sub>CM</sub> numbers, this difference between both mouse strains was limited to pLN (Fig. 4E). CD4<sup>+</sup> T<sub>N</sub> and CD4<sup>+</sup> T<sub>CM</sub> frequencies and numbers were unaltered in pLN and spleens of FRC<sup>+/+</sup> and FRC<sup>−/−</sup> mice (Fig. 4F–H). Similar to CD8<sup>+</sup> T<sub>CM</sub> (Fig. 4D), frequencies of CD4<sup>+</sup> T<sub>CM</sub> were reduced in FRC<sup>−/−</sup> pLN but were only slightly affected in spleens (Fig. 4G). Absolute cell numbers were not significantly different in pLN and spleens of both mouse strains (Fig. 4H). Hence, IL<sub>7</sub> gene inactivation in FRCs is associated with a reduction of CD8<sup>+</sup> T<sub>CM</sub>, an effect that was by far less pronounced for CD4<sup>+</sup> T<sub>CM</sub>.

The survival of both, T<sub>N</sub> and T<sub>CM</sub>, critically relies on IL-7 [1,4] suggesting that incomplete IL7<sup>−/−</sup> gene inactivation or the presence of non-pLN-derived IL-7 created IL-7 levels in FRC<sup>−/−</sup> pLN that were sufficient for T<sub>N</sub> survival but too low for T<sub>CM</sub> maintenance. However, this assumption would predict different efficacies of IL-7 utilization by TN and TCM. Consistent with this idea and recent data [39], IL-7 treatment induced a more efficient IL-7 scramble of FRC<sup>−/−</sup> mice whereas T<sub>CM</sub> appeared to be less dependent on FRC-derived IL-7 (Fig. 4J and K). This finding indicates that FRC-derived IL-7 helps to maintain both, virtual as well as foreign antigen-specific CD8<sup>+</sup> T<sub>CM</sub>.

Discussion

In steady state, IL-7 is supposed to be produced at constant levels [50], mainly by radio-resistant stromal cells [1,51]. T<sub>N</sub> and T<sub>LM</sub> express high levels of the IL-7R enabling them to remove IL-7 from the system continuously [50]. As soon as the peripheral T cell pool reaches a critical size, IL-7 production and consumption reach the equilibrium and the survival of additional T cells is prevented. Hence, the maintenance of T cell homeostasis relies on the competition for limiting amounts of IL-7 [14,50,52].

The seminal work by Link et al. identified LECs and FRCs as main sources of IL-7 in pLN. Additionally, co-culture experiments...
Figure 4. FRC-derived IL-7 determines TCM abundance in pLNs. (A–E) CD3+CD8+ and (F–H) CD3+CD4+ T cells from pLNs and spleens (Sp) of FRCwt (Prx1-Cre+IL-7wt/wt) and FRCΔΔIL-7 (Prx1-Cre+IL-7fl/fl) mice were analyzed for their expression of the indicated molecules. (A, B, C, and F) Shown are representative contour plots and numbers indicate percentages. Bar diagrams represent percentages or numbers of (A) CD44hiBcl-2hi, (B) CD44hiEomeshi cells or (D, E, G, H) T_N (CD44hiCD62Lhi), T_EM (CD44hiCD62Llo), and T_cm (CD44hiCD62Lhi). (A–H) Data in bar diagrams represent pooled results (mean ± SEM) from 10–11 mice combined and analyzed in three independent experiments. (I–K) FRCΔΔIL-7 (Prx1-Cre+IL-7fl/fl) and FRCwt (Prx1-Cre+IL-7wt/wt) mice (all Thy1.2+) received 7 × 10^5 naive CD8+Thy1.1+ OT-I T cells. Twenty-four hours later, recipient mice were vaccinated.
revealed that FRC-derived IL-7 promotes TN survival [9] suggesting that circulating TN receive IL-7-dependent survival signals in pLNs [14–17]. Besides its impact on TN homeostasis, IL-7 also promotes the formation and maintenance of other pLN-homing immune cells including CD8\(^{+}\) T\(_{CM}\) [1,8,40] and ROR\(_{γt}\)\(^{+}\) type 3 innate lymphoid cells (ILC3) [53,54]. Therefore, a common pool of FRC-derived IL-7 is supposed to regulate homeostasis of multiple immune cells in pLNs [17].

There is accumulating evidence that CD8\(^{+}\) TN and TCM pools are regulated independently [55–57] indicating that they colonize different ecological niches [55–57]. In the immune system, ecological niches are defined by the combination of resources affecting the survival and function of a particular immune cell population [57]. In order to limit competition and enable the simultaneous survival of multiple immune cell types, ecological niches must be segregated. However, niche segregation of CD8\(^{+}\) TN and TCM appears to be incomplete as suggested by their common IL-7 dependence [40,56]. Nevertheless, we do not know yet if niche segregation involves the IL-7-dependent spatial separation of both cell types. The uneven distribution of IL-7-producing FRCs [29,58] suggests that, similar to chemokines [59,60], areas of high and low IL-7 density exist in pLNs. Based on their differential IL-7 demands, this assumption would predict the accumulation of CD8\(^{+}\) TN and TCM in separate pLN regions. Of note, the degree of local CD8\(^{+}\) TN and TCM segregation in pLNs varies strongly between experimental systems [61,62]. Whether this context-dependent effect correlates with the presence or absence of IL-7-producing FRCs in particular regions is still unclear since, at least to our knowledge, reliable reagents for IL-7 protein detection in pLNs are still missing.

Although we cannot fully exclude that different anatomical locations modulate distinct aspects of IL-7-dependent CD8\(^{+}\) TN and TCM homeostasis, our results indicate that variable IL-7 sensitivities of CD8\(^{+}\) TN and TCM contribute to the segregation of their ecological niches. In agreement with a recent study [39], we confirmed that IL-7R signaling is less efficient in CD8\(^{+}\) TCM. In a situation of limited IL-7 availability, this property would provide an explanation for the reduction of virtual as well as foreign antigen-specific CD8\(^{+}\) TCM in FRC\(^{IL-7}\) mice. Furthermore, our results are in line with the current paradigm of IL-7-dependent T cell homeostasis proposing that the optimized utilization of limiting IL-7 amounts is prerequisite for the survival of the greatest possible number of IL-7-dependent immune cells [63]. Based on this model, the degree of competition between different IL-7-consuming cells would be restricted and the limited space within pLNs would be used most optimally [14,50].

The insensitivity of TN to FRC-specific I7 inactivation may be due to the fact that TN are anyway capable of surviving short phases of IL-7 deficiency [63]. Indeed, IL-7 binding induces the down-modulation of IL-7R expression by TN more rapidly than by TCM rendering them insensitive to further IL-7 signals [63]. This effect is transient and appears to fulfill at least two functions. First, the amount of IL-7 consumed by a single TN is restricted thereby optimizing IL-7 availability for other immune cells [63]. Second, permanent IL-7R signaling would cause chronic T cell activation and subsequent activation-induced cell death [64]. Keeping in mind that (i) multiple organs produce IL-7 [24,25] and (ii) TN continuously circulate through the body, they may tolerate the partial IL-7-deficiency in FRC\(^{IL-7}\) pLNs because they received critical IL-7 signals elsewhere. Alternatively, incomplete I7 gene inactivation in FRC\(^{IL-7}\) pLNs may allow the production of residual IL-7, which is just sufficient to promote local TN survival. In any case, our data demonstrate that TCM and TN do not tolerate the reduction of IL-7 in FRC\(^{IL-7}\) pLNs equally well. As shown for polyclonal CD8\(^{+}\) T cells in the steady state and for CD8\(^{+}\) OT-I T cells after vaccination, TCM prove to be particularly sensitive to IL-7 ablation in FRCs. However, CD8\(^{+}\) TCM are only partially reduced in FRC\(^{IL-7}\) mice. Whether this is due to the survival of CD8\(^{+}\) TCM subsets with reduced IL-7 demands remains to be shown in the future.

When we compared FRC\(^{IL-7}\) and FRC\(^{−}\) pLNs, we did not observe any obvious differences (Supporting Information Fig. 2A–C). T and B lymphocyte distribution, stromal cell localization, and relative distances between FRCs and lymphocytes appeared normal in FRC\(^{IL-7}\) mice (Supporting Information Fig. 2A–C). Furthermore, chemokine expression was comparable between FRC\(^{IL-7}\) and FRC\(^{−}\) pLNs (Supporting Information Fig. 2D) and ILC3 contributing to the IL-7-dependent regulation of T cell homeostasis [51,54,65] were similarly abundant (Supporting Information Fig. 2E). Hence, our findings argue for normal LN development and function in the absence of FRC-derived IL-7. This strongly suggests that the reduction of TCM in FRC\(^{IL-7}\) results from a lack of IL-7-dependent homing/survival signals rather than structural and/or functional alterations of FRC\(^{IL-7}\) pLNs.

In summary, we provide evidence that IL-7 produced by pLN FRCs regulates T cell homeostasis. As opposed to the current model, our data demonstrate that pLN FRC-derived IL-7 is of limited importance for the local and systemic survival of TN. On the contrary, the maintenance of TCM critically relies on steady state levels of FRC-derived IL-7 suggesting that TN and TCM colonize different ecological niches in vivo.

**Material and Methods**

**Mice**

Prx1-Cre [66] (stock no. 005584) and Lyve1-Cre [30] (stock no. 012601) mice were purchased from The Jackson Laboratory.
Together with IL-7−/− [3], PGK-Cre [27], Flpo [67], Rag1−/−-Thy1.1+ OT-1 [68], and ROSA26RFP [33] mice, they were maintained under specific pathogen-free conditions at the central animal facility of the Medical Faculty of the Otto-von-Guericke-University Magdeburg. Whenever possible, control littersmates were used. Experimental procedures were approved by the relevant animal experimentation committee and performed in compliance with international and local animal welfare legislations (Landesverwaltungsamt Sachsen-Anhalt Permit Number: 42502-2-1288 UnIMD).

**Generation of IL-7fl/fl mice and genotyping**

The C57BL6/N (B6) embryonic stem cell (ES) line JM8A3.N1 harboring the “knockout-first” allele II7^{fl^{m1a}(EUCOMM)Wtsi} was provided by The European Conditional Mouse Mutagenesis Program (EUCOMM). Mice harboring the II7^{fl^{m1a}(EUCOMM)Wtsi} allele were generated by standard blastocyst injection and crossed to Flpo-transgenic mice [67] in order to remove the FRT-flanked part of the targeting construct (Supporting Information Fig. 1A). Resulting mice harboring floxed II7 alleles (II7ff) were crossed to the indicated Cre-transgenic mice in order to delete exons 3 and 4. Mice were genotyped by PCR using the forward primer 5'-AGAGATGCAAGGGACACATCTGGC-3' (upstream FRT site 1), the reverse primers 5'-ATTTTTCTGATTTCACTTACTGCG-3' (upstream exon 3) and 5'-GACGCCGTTTTCTCTGTAGTCC-3' (downstream FRT site 1) exhibiting a 445 bp band for the II7^{fl^{m1a}(EUCOMM)Wtsi} allele, a 680 bp band for the floxed II7 allele, and a 523 bp band for the WT allele.

**Cell isolation**

To obtain single cell suspensions from pLNs and spleens, organs were forced through metal strainers in PBS/2 mM EDTA (Carl Roth) and erythrocytes were lysed. For erythrocyte lysis, spleen cells were forced through metal strainers in PBS/2 mM EDTA (Carl Roth) and erythrocytes were lysed. For erythrocyte lysis, spleen cells were forced through metal strainers in PBS/2 mM EDTA (Carl Roth) and erythrocytes were lysed. To obtain single cell suspensions from pLNs and spleens, organs were forced through metal strainers (Corning, Durham, NC). For LSC and ILC isolation, fat-free pLNs were cut into small pieces in RPMI 1640/10% FCS/1% P/S. Peripheral LN fragments were vortexed and the supernatant was removed (1×). Cells in the supernatant were collected and ILCs were analyzed by flow cytometry. pLN fragments were transferred into 12-well plates containing 1 mL digestion medium I (0.2 mg/mL Collagenase P [Roche], 0.2 mg/mL Dispase II [Roche], 10 µg/mL DNase I [Sigma], and 5 µg/mL Latrunculin B [Calbiochem] in RPMI 1640 supplemented with 10% FCS/1% P/S). After incubation for 30 min at 37°C and 5% CO2, 1 mL digestion medium II (0.4 mg/mL Collagenase P, 0.2 mg/mL Dispase II, 10 µg/mL DNase I, and 5 µg/mL Latrunculin B in RPMI 1640/10% FCS/1% P/S) was added and the samples were re-suspended. After incubation for 30 min at 37°C and 5% CO2, 0.5 mL RPMI 1640/10% FCS/1% P/S/ 10 mM EDTA was added to stop digestion. Cell suspensions were filtered through 70 µm cell strainers and cells were washed with PBS/2 mM EDTA. Cells were resuspended in PBS/2 mM EDTA and filtered through 40 µm cell strainers.

**Adoptive T cell transfer**

Naïve (CD44loCD62lhi) CD8+ T cells (Supporting Information Fig. 6C) expressing a transgenic TCR (Vα2Vβ5) specific for the chicken OVA-derived, H2-Kb-restricted peptide OVA257–264 (SIINFEKL), were isolated from LNs and spleen of Rag1−/− Thy1.1+ OT-1 mice using CD8α-specific MicroBeads and AutoMACS (Miltenyi Biotec) according to the manufacturer’s recommendations. Thy1.2+ recipients received 4–7 × 105 OT-I T cells (purity > 81.7%) via i.v. injection into the tail vein. Twenty-four hours after T cell transfer recipient mice were immunized with a mixture of 50 µg SIINFEKL (Biosyant) and 50 µg PolyI:C (Invivogen).

**In vitro IL-7 stimulation**

Single cell suspensions of peripheral and mesenteric lymph nodes were adjusted to 5 × 106 cells/mL and incubated for 30 min at 37°C and 5% CO2 in RPMI 1640 supplemented with 10% FCS/1% P/S/2 mM L-glutamine (Gibco)/1 mM sodium pyruvate (Gibco)/0.1 mM HEPES (Gibco)/50 µM 2-mercaptoethanol (Sigma) and 1 ng/mL recombinant mouse (protein carrier free) IL-7 (EBioscience, Thermo Fisher Scientific).

**Flow cytometry cell sorting of LSCs**

LSCs were isolated from peripheral and mesenteric lymph nodes as described above. LN cells were incubated with purified anti-CD16/32 (2.4G2 ATCC® HB-197™) in staining buffer (PBS/0.5% [w/v] BSA [AppliChem]/2 mM EDTA) for 10 min at 4°C. Subsequently, cells were stained with biotinylated CD45- and TER-119-specific antibodies in staining buffer containing anti-CD16/32 for 30 min at 4°C. CD45+ and TER-119+ cells were depleted using Dynabeads Biotin Binder (Invitrogen). In brief, cells were re-suspended in staining buffer to 1 × 107 cells/mL and 50 µL pre-washed magnetic beads were added. Cells were incubated for 30 min at 4°C under gentle rotation and CD45+ and TER-119+ cells were removed subsequently using a DynaMag-15 (Thermo Fisher Scientific). Remaining cells were stained with fluorochrome-labeled gp38- and CD31-specific antibodies as well as streptavidin-FITC for 30 min at 4°C. Finally, after washing with PBS/2 mM EDTA, the cells were re-suspended in RPMI 1640/10% FCS/1% P/S. For dead cell exclusion, 7-aminocoumarin D (7-AAD; BioLegend) was added 5 min prior to cell sorting using a FACSARIA III (Becton Dickinson). Live (7-AAD−),
TER-119<sup>−</sup>C4D5<sup>−</sup> LSC subsets were sorted based on their differential gp38/CDS1 expression. Purities of the indicated LSC subsets were >73.3% (data not shown).

**Flow cytometry**

The following reagents were purchased from BioLegend: anti-mouse Bcl-2 (10C4), CD3 (145-2C11), CD3 (17A2), CD4 (RM4-5), CD5 (53-7.3), CD8a (53-6.7), CD11c (N418), CD19 (6D5), CD44 (IM7), CD45 (30-F11), CD62L (MEL-14), CD127 (A7R34), gp38 (8.1.1), Gr1 (RB6-8C5), NK1.1 (PK136), TER-119 (TER-119), Thy1.1 (OX-7), V<sub>o</sub>2 (B20.1), 7-AAD viability staining solution, and streptavidin-FITC. Anti-mouse CD31 (390), Eomes (Dan11mag), and RORγ<sub>t</sub> (B2D) were purchased from ebioscience. Anti-mouse CD45R (B220; RA3-6B2) and the anti-mouse TCR Vβ screening panel were purchased from BD Biosciences. Prior to staining with fluorochrome-labeled antibodies, single cell suspensions were incubated with 50 μL of anti-mouse CD16/32 in staining buffer for 10 min at 4°C. Afterward, cells were incubated with 50 μL of fluorochrome-labeled antibodies diluted in anti-CD16/32 containing staining buffer. After incubation for 30 min at 4°C, cells were washed with 200 μL PBS/2 mM EDTA. For intracellular stainings (except pSTAT5), samples were processed using the FoxP3/Transcription Factor Staining Buffer Set (ebioscience, Thermo Fisher Scientific) according to the manufacturer’s recommendations. For staining of pSTAT5, cell samples were fixed with PBS/2% (w/v) paraformaldehyde (Sigma–Aldrich) and washed with 200 μL Intraacellular Staining Permeabilization Wash Buffer (BioLegend). Subsequently, cells were incubated with anti-mouse pSTAT5 Y694 (47; BD Biosciences) in wash buffer for 30 min at 4°C, washed with 200 μL Wash Buffer and finally resuspended in PBS/2 mM EDTA prior to analysis. For LSC analyses, 7-AAD was added 5 min prior to data acquisition. Samples were measured on a LSRFortessa (Becton Dickinson) and analyzed with FlowJo 9/10 (v9.2.10 software (FlowJo, LLC) according to the “Guidelines for the use of flow cytometry and cell sorting in immunological studies” [69]. Individual gating strategies are depicted in Supporting Information Figs. 1, 2, and 4–6.

**Reverse transcriptase PCR (RT-PCR) and real-time quantitative PCR (RT-qPCR)**

Colon samples were transferred into CK14 2 mL tubes (Peqlab/VWR) containing 700 μL TRIzol reagent (Invitrogen) and homogenized in a Precellys 24 homogenizer (Peqlab/VWR). Peripheral LNs were transferred into CK14 0.5 mL tubes (Peqlab/VWR) containing 200 μL TRIzol reagent and homogenized. Sorted LSCs were re-suspended in 500 μL TRIzol reagent. For RNA extraction, chloroform (Sigma–Aldrich) was added and total RNA was isolated according to the manufacturer’s instructions.

Isolated RNA was quantified by photometric Nanodrop (Thermo Fisher Scientific) measurement. RNA was reverse-transcribed using random hexamer primers and the advantage RT-for-PCR kit (Takara Clontech) according to the manufacturer’s instructions.

For RT-PCR analyses of colon samples, the Taqman<sup>®</sup> Gene Expression Master Mix (Thermo Fisher Scientific) and the following TaqMan<sup>®</sup> Gene Expression Assays (Thermo Fisher Scientific) were used according to the manufacturer’s instructions: Il7 (FAM-MGB probe Mm01295804_m1) and Hprt (FAM-MGB probe Mm00446968_m1). PCR products were analyzed by agarose gel electrophoresis.

For RT-qPCR analyses of sorted LSCs and whole pLNs, the Taqman<sup>®</sup> Gene Expression Master Mix (Thermo Fisher Scientific) and the following TaqMan<sup>®</sup> Gene Expression Assays (Thermo Fisher Scientific) were used according to the manufacturer’s instructions: Ccl19 (FAM-MGB probe Mm00839967_g1), Ccl21 (FAM-MGB probe Mm03646971_gH), Cxcl9 (FAM-MGB probe Mm00434946_m1), Cxcl10 (FAM-MGB probe Mm00445235_m1), Cxcl13 (FAM-MGB probe Mm00444534_m1), Il7 (FAM-MGB probe Mm01295805_m1), and Hprt (FAM-MGB probe Mm00446968_m1). Samples were analyzed in triplicates and Ct values were exported from the ABI PRISM 7000 (Applied Biosystems) sequence detection system. The relative quantifications were calculated according to the ΔC<sub>T</sub> method.

**Automated multidimensional fluorescence microscopy by multi-epitope-ligand cartography**

Multi-epitope-ligand cartography (MELC) was performed as described previously [70]. Briefly, pLNs were embedded into Tissue-Tek<sup>®</sup> O.C.T.<sup>TM</sup> compound (Sakura Finetek), frozen on dry ice, and stored at −80°C. Ten micrometer cryo-sections adhered to silane-coated cover slides (Thermo Fisher Scientific) and washed with PBS/2% (w/v) paraformaldehyde (Sigma–Aldrich), permeabilized with PBS/0.2% (v/v) Triton-X-100 (Carl Roth) and blocked with PBS/1% (v/v) BSA (Sigma–Aldrich) + 30% (v/v) normal goat serum (Invitrogen). Tissue samples were transferred to an inverted wide-field fluorescence microscope (Leica DMi8, 20× air lens NA 0.80; Leica Microsystems). The automated cycled robotic process started with the incubation of the first fluorochrome-labeled antibody (tag). After a series of washing steps, the fluorescence signals and a corresponding phase contrast image were acquired by a cooled charge-coupled device camera (Apogee KX4; Apogee Instruments). The specific signal of the given tag was removed by bleaching the fluorescent dye followed by recording of post-bleaching fluorescence signals and repetition of incubation-imaging-bleaching-cycle. The appropriate working dilutions, incubation times, and positions within the MELC experiment of the used tags (anti-mouse CD3 (17A2), CD31 (390), CD8a (53-6.7), gp38 (8.1.1), CD45 (30-F11), CD54 (YN1/1.7.4), CD44 (IM7), and CD45R/B220 (RA3-6B2) were purchased from BioLegend, anti-mouse CD4 (RM4-5) from BD Biosciences, PI from Sigma–Aldrich) were validated systematically using conditions suitable to MELC [70]. The series of fluorescence images produced by each tag were aligned pixel-wise using the corresponding phase
Statistical analyses

Statistical analyses and graphical representations were performed using Prism 5.0d/f (GraphPad Software Inc.). Statistical significances were determined using non-parametric two-tailed Mann–Whitney U tests; \( * p \leq 0.05; \quad ** p \leq 0.01; \quad *** p \leq 0.001; \quad **** p \leq 0.0001.\)

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