Hoxb4 Overexpression in CD4 Memory Phenotype T Cells Increases the Central Memory Population upon Homeostatic Proliferation

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Abstract

Memory T cell populations allow a rapid immune response to pathogens that have been previously encountered and thus form the basis of success in vaccinations. However, the molecular pathways underlying the development and maintenance of these cells are only starting to be unveiled. Memory T cells have the capacity to self renew as do hematopoietic stem cells, and overlapping gene expression profiles suggested that these cells might use the same self-renewal pathways. The transcription factor Hoxb4 has been shown to promote self-renewal divisions of hematopoietic stem cells resulting in an expansion of these cells. In this study we investigated whether overexpression of Hoxb4 could provide an advantage to CD4 memory phenotype T cells in engrafting the niche of T cell deficient mice following adoptive transfer. Competitive transplantation experiments demonstrated that CD4 memory phenotype T cells derived from mice transgenic for Hoxb4 contributed overall less to the repopulation of the lymphoid organs than wild type CD4 memory phenotype T cells after two months. These proportions were relatively maintained following serial transplantation in secondary and tertiary mice. Interestingly, a significantly higher percentage of the Hoxb4 CD4 memory phenotype T cell population expressed the CD62L and Ly6C surface markers, characteristic for central memory T cells, after homeostatic proliferation. Thus Hoxb4 favours the maintenance and increase of the CD4 central memory phenotype T cell population. These cells are more stem cell like and might eventually lead to an advantage of Hoxb4 T cells after subjecting the cells to additional rounds of proliferation.

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Introduction

Memory T cells develop from a small subset of effector T cells following a primary immune response. While effector T cells undergo apoptosis, memory T cells survive and provide the host an immunological memory allowing a faster and more effective immune response against previously encountered pathogens. Memory T cells are long-lived cells and their survival after antigen clearance depends on the homeostatic cytokines interleukin (IL)-7 and IL-15 [1–5]. Memory T cells persist by undergoing a slow turn-over, also referred to as basal homeostatic proliferation, with a frequency of one division in 2–3 weeks [3]. However, upon transfer into a lymphopenic host, memory T cells divide rapidly due to an increased availability of IL-7 and IL-15 [1–6], a phenomenon indicated as acute homeostatic proliferation. Knock-out mouse models for IL-15, IL-7 and IL-7Rα signalling demonstrated that CD4 and CD8 memory T cells have a differential dependence for these cytokines. In the absence of IL-15 the basal homeostatic proliferation of CD8, but not CD4 memory T cells was severely reduced [1,7,8], while CD4 memory T cells fail to persist upon transfer into IL-7 deficient hosts [9]. However, acute homeostatic proliferation of both CD4 and CD8 memory T cells can be induced by either IL-15 or IL-7Rα signalling [1,3,10]. In addition to IL-7 and IL-15, which are the key factors for the survival and homeostatic proliferation of memory T cells, other cytokines have been shown to boost their homeostatic proliferation, such as IL-2 and interferon-1 (IFN-I) [11–13]. Despite their independence for T cell receptor (TCR) signalling to survive, experiments using knock-out mice showed that antigen specific CD4 memory T cells had reduced responses to antigen re-encounter in the absence of major histocompatibility complex (MHC) II [14]. Moreover, the presence of MHC II signals influenced the homeostatic expansion capacity of memory T cells under lymphopenic conditions, but this appeared to be independent on the avidity for MHC II, in contrast to naive T cells [15]. This suggests that regulatory mechanisms governing memory homeostasis are different from naive T cell homeostasis, which is important to maintain optimal diversity of the memory pool.
In addition to antigen-experienced memory T cells (true memory) a population of immunophenotypically identical memory cells exists that arise from interactions of the T cell receptor with endogenously expressed antigens [16] and are also referred to as memory phenotype (MP) T cells. Similarly to antigen-experienced memory cells, MP T cells are proliferating in response to lymphopenia and at least for CD8 it has been shown that they provide protection against antigen [3,17,18].

The requirements for homeostatic proliferation of MP T cells are slightly different than for true memory T cells. In addition to IL-15 and IL-7 they are dependent on MHCII [3,19,20], likely to avoid competition for signals provided in the niche.

Despite our increasing knowledge on the required signals, the molecular pathways behind homeostatic proliferation are still elusive. Some transcription factors have been shown to induce the expression of IL-7R or CD122 and thus allowing their permissive state to homeostatic survival and proliferation signals. For example, Foxo1 and GABPa promote IL-7R expression in T cells [21,22]. In contrast, transcription factor Gfi-1 downregulates IL-7R expression by inhibiting GABPa following TCR signalling or cytokine stimulation [23]. On the other hand, transcription factors T-bet and Eomes are found to maintain high levels of CD122 on CD8 memory T cells [24]. In addition, genes encoding for epigenetic regulators of transcriptional programs have been attributed important, but distinct functions in Th2 memory cells. First, using a knock-out model it was demonstrated that the polycomb gene Bmi1 is critical for the survival of CD4 memory T cells through repression of the pro-apoptotic gene noxa [25]. Furthermore, the trithorax gene MLL was shown to provide activating histone modifications on the GATA3 and Th2 cytokine.
loci, which are required for Th2 memory function [26,27]. Interestingly, both MLL and Bmi1 are also critical for the maintenance of hematopoietic stem cells (HSC) [28–30]. Actually, memory T cells and HSCs have several features in common, such as longevity, the ability to self-renew at a very low rate normally followed by re-entering quiescence and the potential to proliferate and differentiate upon cytokine or antigen receptor signalling. Moreover, an overlap was observed in gene expression patterns between memory T, B cells and long-term HSCs [31], supporting the fact that identical molecular pathways might be involved in self-renewal of HSCs and memory T cells.

Hoxb4 is another well known critical regulator of HSCs and belongs to the family of homeobox (Hox) genes, which are transcription factors initially found to determine cell fate in the embryo [32]. The expression of Hox genes is epigenetically regulated by the antagonistic actions of polycomb and trithorax genes [33]. Hoxb4 is expressed in HSCs, but knock-out mouse models for Hoxb4 showed that Hoxb4 is not essential for their generation [34,35]. However, retroviral mediated overexpression of Hoxb4 in bone marrow (BM) cells resulted in the expansion of HSCs through promotion of self-renewal divisions without development of overt leukemia in BM chimeric mice [36,37]. Interestingly, like memory T cells HSCs do not persist in the absence of Bmi1 [28,29]. However, Hoxb4 overexpression could not rescue the long-term maintenance of HSCs deficient for Bmi1, despite the triggering of self-renewal of Bmi1−/− HSCs [30]. Thus while Hoxb4 has a function in the execution of the self-renewal division, Bmi1 provides the HSCs their sustainability. With respect to the resemblances between HSCs and memory T cells, it is likely that these distinct functions might also apply to memory T cells. In this study we set out to evaluate whether Hoxb4 overexpression would lead to an enhanced self-renewal activity of CD4 MP T cells. Using Hoxb4 lymphoid specific transgenic mice, we investigated the acute homeostatic proliferation of Hoxb4 CD4 MP T cells and wild type (wt) MP T cells following transfer in competition into lymphopenic mice. Surprisingly, Hoxb4 did not provide CD4 MP T cells with an advantage in repopulating the empty T cell niche. In fact the overall contribution of Hoxb4 MP T cells was significantly lower, but remained rather stable after two additional rounds of homeostatic proliferation. Intriguingly, Hoxb4 MP T cells consistently comprised a significant larger population of cells expressing surface markers CD62L and Ly6C, indicating a central memory T cell phenotype.

### Materials and Methods

**Ethics Statement**

All animal experiments have been performed in accordance with the guidelines of the Canadian Council on Animal Care and have been approved by the Hospital Maisonneuve–Rosemont animal protection committee (protocol number 2012–20).

**Mice**

Hoxb4 transgenic mice were generated using the pLIT3 vector (Fig. 1A) and have been described by us before [39]. PCR for the hGH gene was performed on genomic tail DNA to identify transgenic mice. Lymphopenic Cd3ε−/− recipient mice for transplantation assays have been originally generated by Malissen et al. [40]. Wt mice C57BL/6 (CD45.2) and B6.SJL (CD45.1) were purchased from Jackson Laboratories (Bar Harbor, ME,

| Table 1. Percentage of T cell populations in hematopoietic organs of young adult mice. |
|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|
| Hoxb4 Gated on CD4 or CD8 cells            | CD4 T cells                                 | CD44loCD62Lhi CD44hi CD44hiCD62Lhi CD44hiCD62Llo CD44hiLy6C+ |
|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|
|                                             | n Total                                    | CD44loCD62Lhi | CD44hi | CD44hiCD62Lhi | CD44hiCD62Llo | CD44hiLy6C+ |
| CD4 T cells                                 |                                             |                                                               |                                                               |                                                               |                                                               |                                                               |
| LN                                         | 5                                           | 36.99±4.47 | 71.49±13.87 | 9.73±4.69 | 2.18±0.80 | 6.06±2.83 | 0.14±0.05 |
| Spleen                                     | 7                                           | 21.68±8.33 | 59.22±14.66 | 24.56±10.32 | 4.29±0.67 | 20.59±11.18 | 0.80±0.37 |
| BM                                         | 7                                           | 1.86±1.05 | 11.51±9.97 | 57.19±8.37 | 6.65±4.71 | 50.54±6.90 | 11.37±7.92 |
| CD8 T cells                                 |                                             |                                                               |                                                               |                                                               |                                                               |                                                               |
| LN                                         | 5                                           | 24.10±8.05 | 52.73±25.58 | 14.83±4.75 | 9.64±4.56 | 4.55±3.87 | 10.47±3.07 |
| Spleen                                     | 7                                           | 10.56±4.93 | 56.91±9.64 | 23.24±4.18 | 16.38±2.88 | 7.46±4.56 | 15.06±1.98 |
| BM                                         | 7                                           | 1.99±1.34 | 25.94±19.95 | 41.68±13.84 | 14.23±9.19 | 27.44±18.25 | 22.16±6.35 |
| Wild Type Gated on CD4 or CD8 cells         | CD4 T cells                                 | CD44loCD62Lhi CD44hi CD44hiCD62Lhi CD44hiCD62Llo CD44hiLy6C+ |
|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|
|                                             | n Total                                    | CD44loCD62Lhi | CD44hi | CD44hiCD62Lhi | CD44hiCD62Llo | CD44hiLy6C+ |
| CD4 T cells                                 |                                             |                                                               |                                                               |                                                               |                                                               |                                                               |
| LN                                         | 6                                           | 38.05±7.45 | 55.80±19.07 | 10.93±3.90 | 2.23±0.57 | 8.69±3.98 | 0.17±0.09 |
| Spleen                                     | 7                                           | 20.50±5.57 | 56.07±12.47 | 22.17±6.24 | 4.56±1.44 | 18.99±5.19 | 1.02±0.48 |
| BM                                         | 7                                           | 1.68±0.80 | 15.30±10.15 | 56.68±17.49 | 6.56±4.62 | 50.13±14.97 | 17.89±11.75 |
| CD8 T cells                                 |                                             |                                                               |                                                               |                                                               |                                                               |                                                               |
| LN                                         | 6                                           | 22.60±9.60 | 38.45±20.26 | 12.36±4.58 | 6.03±2.95 | 6.33±5.14 | 9.17±3.89 |
| Spleen                                     | 7                                           | 9.36±4.31 | 52.06±10.42 | 19.90±4.83 | 12.72±2.89 | 7.52±3.23 | 13.53±2.64 |
| BM                                         | 7                                           | 1.75±1.65 | 30.01±21.76 | 34.78±15.10 | 12.41±6.61 | 22.37±12.93 | 20.60±6.32 |

Note that no significant differences were observed between T cell populations of Hoxb4 and wild type mice. 1-tailed Student ttest, comparing Hoxb4 vs. wild type mice.

LN = Lymph node; BM = bone marrow.

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Figure 2. Change of naive and MP T cell populations in Hoxb4 transgenic and wt mice with age. Scatter plots showing the percentage of (A) CD4 and (B) CD8 T cells that are naive (CD44lo/CD62Lhi), MP (CD44hi) or a subpopulation of MP T cells (CD44hi/Ly6Chi) for LN, Spl and BM.
Hoxb4 transgenic mice (CD45.2) were bred to B6.SJL mice to generate compound CD45.1/2 Hoxb4 transgenics. Mice were housed at the animal facility of the Maisonneuve-Rosemont Hospital Research Center under specific pathogen free conditions.

**FACS Analysis and Sorting**

To analyze memory T cell populations the following antibodies with conjugated fluorochromes were used: CD8α-PerCP, CD62L-Pacific Blue, Ly6C-Alexa647, Sca-1-PE/Cy7 or Pacific Blue (all obtained from BioLegend, San Diego, CA, USA); CD4-APC/Cy7, CD44-PE/Cy7 or -APC, Ly6C-FTTC, CD62L-PE-Cy7 (BD Pharmingen, Mississauga, ON, Canada); CD127-biotin and CD62L-EFluor® 605NC (eBioscience, San Diego, CA, USA). To determine the transgenic or wt origin of MP T cell populations in competitive transplantation assays following antibodies against the CD45 alleles were used: CD45.1-Pacific Blue or -FITC (BioLegend) and CD45.2-APC or -V500 (BD Bioscience). Labelled cells were analyzed on a LSR II with an UV laser (BD Bioscience) and further analyzed using the Diva software. FlowJo software (TreeStar Inc., Ashland, OR, USA) was used to further analyze specific cell populations.

MP T cell populations for transplantations assays or expression analysis were sorted on a FACSaria III sorter (BD Bioscience) using antibodies for CD45-APC (BioLegend) or -PE/Cy7 (BD Bioscience), CD4 -PerCP, CD8α-PerCP, CD25-biotin and NK.1.1-biotin, all from BD Bioscience. Biotin conjugated antibodies were detected with Streptavidin-PE (BioLegend). MP T cells were gated on either CD4+ or CD8+ and further defined as CD44hi/CD25- /NK.1.1-.

**Competitive Transplantation Assays**

CD4 MP T cells were sorted from spleen and lymph nodes (LNs) of 3 to 4 months old Hoxb4 transgenic (CD45.1/2) and congeneric wt (CD45.1) mice. A cell dose of 2 x 10⁶ cells composed of equal numbers of Hoxb4 and wt MP T cells were transplanted in sex matched CD3ec−/− mice by injection in the tail vein. For serial transplantation, mice were sacrificed two months post-transplantation and 10⁷ cells derived from the LNs of each donor were transplanted into a secondary CD3ec−/− host. A third transplantation was repeated again after two months.

For evaluation of short-term proliferation under competitive conditions, sorted Hoxb4 and wt MP T cells were labelled with CellTrace® Violet (Life Technologies Inc.) according to the

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**Table 2. Percentage of T cell populations in hematopoietic organs of aged adult mice.**

| Hoxb4 | Gated on CD4 or CD8 cells | n | Total | CD44hiCD62Lhi | CD44hi | CD44hiCD62Lhi | CD44hiCD62Llo | CD44hiLy66C+ |
|-------|--------------------------|---|-------|----------------|--------|----------------|----------------|--------------|
| CD4 T cells | | | | | | | | |
| LN | 5 | 20.44±5.41 | 19.98±16.36 | 60.76±21.87 | 4.39±1.85 | 56.37±21.20 | 0.38±0.19 |
| Spleen | 7 | *11.43±3.09 | 6.08±7.47 | 73.77±15.76 | 3.05±1.85 | 71.76±15.40 | 1.16±0.35 |
| BM | 7 | *2.91±1.60 | *2.28±1.61 | 53.03±34.94 | 1.32±0.86 | 52.22±31.56 | 6.06±6.97 |
| CD8 T cells | | | | | | | | |
| LN | 5 | 13.78±7.82 | 19.07±15.09 | 63.75±22.69 | 30.64±14.72 | 33.11±16.19 | 33.71±16.88 |
| Spleen | 7 | *4.68±1.96 | 16.02±16.12 | 56.93±32.64 | 17.09±8.75 | 42.17±24.90 | 28.65±14.06 |
| BM | 7 | *1.51±0.98 | 8.45±12.25 | 63.91±27.87 | 10.23±7.45 | 50.98±21.90 | 19.32±10.93 |
| Wild Type | | | | | | | | |
| CD4 T cells | | | | | | | | |
| LN | 6 | 22.49±7.55 | 16.74±7.90 | 57.35±20.85 | 5.64±3.04 | 51.72±17.96 | 0.56±0.24 |
| Spleen | 7 | 18.12±5.57 | 6.94±9.65 | 74.77±16.12 | 2.98±1.07 | 71.79±15.60 | 1.33±0.47 |
| BM | 7 | 5.55±1.50 | 0.76±0.44 | 67.47±14.67 | 2.01±1.22 | 65.47±14.44 | 8.14±7.67 |
| CD8 T cells | | | | | | | | |
| LN | 6 | 17.38±10.71 | 12.88±8.31 | 63.46±26.18 | 37.33±26.09 | 26.15±8.15 | 37.87±16.45 |
| Spleen | 7 | 9.26±5.48 | 9.25±10.95 | 60.09±32.16 | 25.22±11.54 | 42.91±10.96 | 28.36±7.18 |
| BM | 7 | 3.60±2.06 | 2.41±3.74 | 72.30±12.48 | 11.76±9.39 | 60.54±12.22 | 17.50±7.77 |

Note the decrease in total T cells in bone marrow and spleen of Hoxb4 mice; *P<0.05;
1-tailed student t test, comparing Hoxb4 vs. wild type mice. LN = Lymph node; BM = bone marrow.

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manufacturer's protocol prior to transplantation. Each recipient received a total dose of $7 \times 10^5$ cells. Mice were sacrificed after one week for analysis.

**Cytokine Analysis**

The production of cytokines was measured as described previously [41]. Splenocytes were stimulated with PMA/ionomycin (5 μg/ml) for 2 hours at 37°C, followed by 2 hours incubation with 100 μg/ml Brefeldin A (Sigma-Aldrich Co., St. Louis, MO) to block cytokine secretion. After fixation with formaldehyde (2%) followed by permeabilization with 0.5% saponine (Sigma-Aldrich) cells were stained with antibodies against cytokines.

**Quantitative Reverse Transcriptase (Q-RT)-PCR**

Total RNA was isolated by Trizol®, DNase-I-treated and cDNA was prepared using MMLV-RT according to the manufacturer's instructions (Invitrogen, Paisley U.K.). Q-RT-PCR was carried out using SYBRGreen® Power mix (Applied Biosystems, Toronto, ON, Canada), using oligonucleotides for Hoxb4 and Gapdh as designed before [42,43]. Reactions were carried out in triplicate. CT-values were corrected for Gapdh.

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**Figure 3. Competitive short-term homeostatic proliferations (7 days) of Hoxb4 transgenic and wt CD4 MP T cells.** (A) Scheme of the experimental approach. CD4 MP T cells are sorted from CellTrace™ Violet (CTV) labelled cells isolated from LN and Spl of Hoxb4 (CD45.1/2) and congenic wt (CD45.1) mice. Cells of both genotypes are transplanted in a 1:1 ratio in CD3e−/− (CD45.2) mice. (B) FACS profiles showing Hoxb4 and wt fractions to donor derived CD4 MP T population (CD45.1) in LN (left panel). Representative FACS profiles for CD62L and CTV on Hoxb4 and wt populations. Loss of CTV tracer indicates that most cells are dividing rapidly (right panels). (C) Average contribution (%) of Hoxb4 and wt cells to donor derived MP T cells in LN, Spl and BM (n = 3). (D) Percentage of CD62Lhi MP T cells in Hoxb4 and wt population found in lymphoid organs. *P<0.05; paired 2-tailed Student t-test. Wt = wild type, MP = memory phenotype, LN = lymph node, Spl = spleen and BM = bone marrow.

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expression and to the expression in a calibrator comprised of BM, spleen and LN cells (ΔACT). The average fold difference over the expression in the calibrator sample was calculated as 2^−ΔΔCT). To compare the expression of Hoxb4 in transgenic and wt mice, the average fold difference of Hoxb4 transgenic was divided by those for the wt mice.

Results

To evaluate whether overexpression of Hoxb4 modulates the size of the MP T cell population, a lymphoid specific transgenic mouse for Hoxb4 was analysed. No major abnormalities in the thymic and splenic lymphoid populations of these mice have been reported [39], but the MP T cell populations have not been analysed in these mice before. To validate the expression of the transgene in MP T cell populations, Q-RT-PCR for these mice before. To validate the expression of the transgene in splenic lymphoid populations of these mice have been reported from LN and spleen of adult Hoxb4 transgenic mice. Both naive and MP T cell subpopulations in aged wt mice were dramatically reduced in LN, spleen and BM (Fig. 1 and Table 1 and 2), while the CD44hi memory populations and their subpopulations CD44hi/Ly6Ghi were increased, except in the BM for CD4. In Hoxb4 transgenic mice the decrease in both CD4 and CD8 T cells with age was more pronounced than in wt mice, reaching significance in both LN and spleen (Fig. S1). Similarly as in wt mice, the proportions of naive CD4 and CD8 subpopulations in old Hoxb4 transgenic mice were decreased compared to young mice and those of CD44hi memory phenotype T cells increased (Fig. 1B; Table 1 and 2). However, the cellularity in hematopoietic organs tended to be reduced in old Hoxb4 mice (Table S1). This had as consequence that the numbers of CD44hi MP T cells were not significantly increased in these mice. Together, these data show that Hoxb4 does not affect T cell homeostasis in young mice that are in steady-state hematopoiesis.

Short-term Competitive Homeostatic Proliferation of CD4 Memory T cells

Adoptive transfer of T cells into a lymphopenic host results in a rapid proliferation of these cells to fill the empty niche. To test whether Hoxb4 CD4 MP T cells have an advantage over wt cells in the initial proliferation phase following transfer, CD4 MP cells (CD44hi/CD25−NK1.1−) were sorted from both Hoxb4 transgenic (CD45.1/2) and wt congenic mice (CD45.1), stained with CellTraceTM Violet (CTV) and transplanted in a 1:1 ratio into CD3e−/− mice lacking T cells (Fig. 3A). One week post-transplantation, the mice were sacrificed and analysed for the presence of donor cells by fluorescence activated cell sorting (FACS). Distinct populations of donor cells were detected in all organs representing 0.20% of the total cell population in both LNs and spleen and 0.03% in BM (data not shown). Hoxb4 and wt CD4 MP T cells contributed equally to LNs and BM of the recipient mice, but in the spleen the wt cells dominated over Hoxb4 MP T cells (Fig. 3B left panel and 3C). Both Hoxb4 and wt MP T cells underwent several divisions during seven days, because the majority of cells were negative for CTV (Fig. 3B).

Interestingly, the proportions of cells expressing the surface marker CD62L, which is characteristic for central memory T cells, were higher in Hoxb4 CD4 MP T cells (Fig. 3D).

Together these data show that both Hoxb4 and wt CD4 MP T cells have divided rapidly to occupy the niche in T cell deficient mice with fluctuations in the different organs. Moreover, overexpression of Hoxb4 promoted the enrichment of CD62L positive cells following lymphopenia induced proliferation.

Medium-term Competitive Repopulation in a Lymphopenic Host

To evaluate whether Hoxb4 CD4 MP T cells would dominate over the wt MP T cells with time, equal numbers of Hoxb4 (CD45.1/2) and wt (CD45.1) CD4 MP T cells were transplanted in CD3e−/− mice and sacrificed after two months. No signs of disease were observed in these mice. At this time point the transferred MP T cells occupied at average 6.6±3.1% of the cells in LN, 4.5±1.7% in the spleen and 0.7±0.8% in the BM of recipient mice (Table 3). This is equivalent with a total of ~6.4×106 donor cells in these organs, which implies that MP T cells have expanded at least 51-fold over the number of injected cells.
The average contribution of wt CD4 MP T cells to the repopulation of the BM and spleen in nine individual recipients transplanted in three independent experiments was 2- to 3-fold higher in the spleen and BM than that of Hoxb4 CD4 MP cells (Table 3 and Fig. 4A and B). However, the percentage of the Hoxb4 and wt CD4 MP T cells in the LNs of host mice was very variable and not significantly different (Fig. 4B and Table 3). Total Hoxb4 and wt CD4 MP T cells in the combined organs were calculated and showed a net expansion of 20-fold and 43-fold for Hoxb4 and wt cells, respectively (Fig. S2A). This resulted in a net 2-fold higher contribution of wt CD4 MP T cells over Hoxb4. Both Hoxb4 and wt CD4 MP T cell populations were further characterized for the presence of specific surface markers such as CD62L, Ly6C, CD127 and 1B11. FACS analysis showed that the...
percentage of Ly6C<sup>+</sup> cells was 3- to 4-fold higher in the Hoxb4 population compared to wt in all organs analysed (Fig. 4C and D). Moreover, in contrast to a negligible proportion of wt CD4 memory T cells that express CD62L (1.0% in all organs), a distinct proportion of Hoxb4 cells expressed this marker in BM (13.9±7.1%), spleen (3.4±1.3%) and LN (3.3±1.2%), indicating that Hoxb4 favours central memory characteristics. All CD62L<sup>+</sup> cells carried also the Ly6C marker (data not shown). The percentage of cells expressing CD127, CD43 and CD44 was not different for both genotypes. Since central memory T cells are considered the long-term memory T cells, we analysed if Hoxb4 overexpression act more specifically on the expansion of CD4 central MP T cells. The total numbers of CD62L<sup>+</sup> cells in BM, spleen and LNs of the Hoxb4 population ranged from 7000 to

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**Figure 5. Long-term competitive homeostatic proliferations (180 days) of Hoxb4 transgenic and wt CD4 cells.** (A) Scheme of serial transplantations. 10<sup>6</sup> cells of the LNs of primary hosts that received a transplant composed of equal doses of Hoxb4 and wt MP T cells were serially transplanted into secondary and tertiary hosts with a 60 days interval. (B) Compilation of Hoxb4 and wt fractions of donor derived cells in LN, Spl and BM of secondary (n = 6) and tertiary hosts (n = 4) from two independent experiments. (C) Bar graphs showing the average percentage of cells positive for CD62L and Ly6C within the Hoxb4 or wt memory T populations. *P<0.05, 2-tailed Student t-test. Wt = wild type, MP = memory phenotype, LN = lymph node, Spl = spleen and BM = bone marrow.

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that in the majority of mice CD62L<sup>+</sup> CD4 MP T cells in the initial graft were considered equal based on data in Table 1 and estimated at 20000 cells. After competitive homeostatic proliferation the number CD62L<sup>+</sup> cells had increased in the *Hoxb4* population in four out of six mice. In contrast the CD62L<sup>+</sup> MP T cells were decreased in the wt population for all six mice. In addition to the molecular make-up of the MP T cells, the functional response after stimulation with PMA was measured. The proportion of *Hoxb4* MP T cells that produced TNF-α and IL-2 was somewhat, but not significantly, reduced. However, a modest, but significant decrease in the number of *Hoxb4* cells producing IFN-γ was observed (Fig. 4E). Thus these data show that in the majority of mice *Hoxb4* CD4 MP T cells were less competitive, but functional. Furthermore, the subset of more central memory like CD62L<sup>+</sup> positive cells was larger in the *Hoxb4* MP T cells and in the presence of *Hoxb4* this population was actually expanded in some mice over the initial transplanted cells.

### Evaluation of Long-term Competitive Repopulation in Lymphopenic Hosts by Serial Transplantation

Central memory T cells have a more extended life span than effector memory T cells and are considered the long-term memory cells. The *Hoxb4* CD4 MP T population comprised a higher number of CD62L<sup>+</sup> memory T cells than the wt after one round of homeostatic proliferation. To evaluate whether the contributions of *Hoxb4* and wt MP T cells changed in favour of *Hoxb4* after several rounds of homeostatic proliferation, 10<sup>6</sup> cells of total LN isolated from six primary hosts were serially transplanted (Fig. 5A), which comprises between 0.5 and 1.0×10<sup>6</sup> donor CD4 MP T cells. The percentage of transferred MP T cells detected in the hematopoietic organs of the secondary hosts after two months of expansion was not significantly different than in primary hosts (Table 3). Evaluation of the proportions of *Hoxb4* and wt MP T cells by FACS showed that the average contribution of *Hoxb4* MP T cells was significantly lower than that of wt in all organs (Fig. 5B and Table 3). However, only in one out of six mice the contribution of *Hoxb4* was higher than that of wt MP T cells in LN and BM. These ratios of *Hoxb4* vs. wt MP T cells were maintained upon transfer and expansion in a tertiary host. Interestingly, the proportion of CD62L<sup>+</sup> and Ly6C<sup>+</sup> cells remained consistently higher in the *Hoxb4* CD4 MP T cell population following serial transfer (Fig. 5C and D). This translated in an actual higher number of *Hoxb4* than wt CD62L<sup>+</sup> cells in five of six secondary hosts (data not shown). Cytokine analysis following stimulation in vitro showed that even after several rounds of expansion *Hoxb4* MP T cells were functionally intact (Fig. S3). Thus despite an increased population of CD62L<sup>+</sup> MP T cells, the contribution of *Hoxb4* CD4 MP T cells did not change after three rounds of expansion.

### Discussion

Memory T cells are triggered to proliferate following the transplantation into a lymphopenic host through increased availability of IL-7 and likely IL-15 [3,6]. In this study we investigated whether *Hoxb4*, known for its potential to expand HSCs, also could increase the pool of CD4 MP T cells. Competitive transplantation of *Hoxb4* transgenic and wt CD4 MP T cells showed that *Hoxb4* overexpression did not provide the total MP T cell population with a proliferative advantage. On the contrary, the overall population of *Hoxb4* MP T cells were less competitive in expansion to occupy the empty niche of CD3ε<sup>−/−</sup> mice than wt CD4 MP T cells. The disadvantage became already apparent in the spleen after 7 days of homeostatic proliferation, despite the fact that most *Hoxb4* CD4 MP T cells had undergone a rapid proliferation (Fig. 3B), and was even more pronounced after 2 months. Two additional rounds of homeostatic proliferation did not further change the established ratios between *Hoxb4* and wt MP T cells. We cannot completely exclude that *Hoxb4* CD4 MP T cells were lost by apoptosis, but we think that this is not very likely, because no differences in apoptosis within the MP T cell populations were observed in *Hoxb4* transgenic and wt mice (data not shown).

A major observation is the enrichment of CD62L<sup>+</sup> and Ly6C<sup>−</sup> subpopulations in the presence of *Hoxb4* overexpression. The CD62L<sup>+</sup> molecole is a classical indicator for central memory T cells [45]. Although Ly6C<sup>+</sup> has been recognized as a marker for CD8 central memory T cells, this is less clear for CD4 memory T cells. Tokoyoda et al. demonstrated that antigen specific CD4 memory T cells preferentially reside in the BM and express high levels of Ly6C<sup>+</sup>, but these cells did not express central memory molecules CD62L or CCR7 [46].

The Ly6C expression on CD4 memory T cells still remains controversial as a more recent study by Marshall et al. demonstrates that Ly6C<sup>+</sup> effector cells persisted better during the contraction phase than Ly6C<sup>+</sup> cells and thus are more prone to develop into memory T cells [47]. In the same study they show that the transcriptional profile of Ly6C<sup>+</sup> effector T cells resembled those of memory T cells. Interestingly, high expression of Ly6C was observed once the effector T cells were converted into memory T cells. It is thus not clear whether Ly6C<sup>+</sup> cells could be considered central memory cells. We found that CD62L expressing cells expressed also Ly6C, but in addition Ly6C<sup>−</sup>/CD62L<sup>−</sup> cells were present. The expression of Ly6C was mostly low to intermediate, while only a small fraction of cells were Ly6C<sup>−</sup> and thus does not contradict observations mentioned by Marshall et al. It is of interest to note that we observed highest proportions of Ly6C and CD62L cells in the BM, which has been allocated as the principal niche for long-term CD4 memory T cells [46]. The actual increase of the CD62L population by *Hoxb4* found in several mice could be achieved by promotion of self-renewal divisions as has been suggested for *Hoxb4* [36]. Thus our data suggest that *Hoxb4* might indeed favour self-renewal of CD4 MP T cells, but only the stem cell like CD62L<sup>+</sup> central memory T cells. Alternatively, *Hoxb4* could activate CD62L or Ly6C<sup>+</sup> expression, however, no binding sites for Hoxb4 or its cofactor Pbx are predicted on the promoter sequences of either gene according to the DECipherment Of DNA Elements (DECODE) database, which compiles predicted binding sites for over 200 transcription factors, suggesting no direct activation. Indirect activation of CD62L by Hoxb4 or through binding to more distant transcription factors, suggesting no direct activation. Indirect activation of CD62L by Hoxb4 or through binding to more distant enhancer regions cannot be excluded, but is unlikely as populations of CD4<sup>+</sup>/CD62L<sup>+</sup> in transgenic *Hoxb4* mice are not enhanced compared to wild type.

In our experimental design, memory T cells were purified from non-immunized mice and are considered MP CD4 T cells that have been generated in the absence of antigen during homeostatic proliferation. It is well known that MP CD4 T cells are a heterogenous population of cells [3]. A subset of these cells has been shown to divide more rapidly than antigen specific memory T cells. This proliferation appeared to be independent of homeostatic cytokines, but these MP CD4 cells do require contact with MHCII, possibly loaded with foreign antigens, for their homeostatic proliferation. It has been reported that these fast dividing CD4 MP T cells have some properties of effector cells [3] and it is thus possible that *Hoxb4* is not favouring the expansion of this subpopulation. A potential reason for the reduced proliferation of the *Hoxb4* CD4 MP T cells might be an effect of *Hoxb4* on
thymic differentiation and the TCR repertoire. However, we did not observe any anomalies of thymic T cell differentiation as thymic FACs profiles showed a normal distribution of thymic cell subsets [39], indicating that Hoxb4 does not interfere with T cell development and thus making this possibility less likely. Furthermore, analysis of the TCR VB usage by peripheral T cells did not show any difference in repertoire between wt and Hoxb4 transgenic mice (data not shown), indicating that a change in TCR repertoire is probably not the reason for the reduced homeostatic expansion of Hoxb4 CD4 MP T cells in our experiments.

Based on the enrichment of CD4 central MP T cells in the Hoxb4 population it was expected that Hoxb4 MP T cell would dominate the repopulation lymphopenic hosts. It is not entirely clear why after three rounds of homeostatic proliferation the contribution of Hoxb4 CD4 MP T cells did not increase. Recently, the gut has been identified as an important reservoir for CD4 memory T cells [48]. One possibility is that Hoxb4 CD4 MP T cells preferentially migrated to the gut site and were not included in our analysis. This requires the expression of adhesion molecule, integrin α4β7. Interestingly, the DECODE database mentions binding sites for Hoxa9 and Meis1 in the promoter of the sigα4 and itgb7 genes coding for integrin α4 and β7, respectively. Although Hoxb4 is not included in their list it is possible that Hoxb4 might target this gene as well, either directly or through complex with Pbx and Meis1. Of note is that Hoxb4 also has the potential to expand HSCs [49], and another integrin sharing the same alpha chain, α4β1 (VLA-4) is expressed on HSCs, allowing for adhesion to the BM stroma [50]. Thus an increase in α4β7 expression on Hoxb4 CD4 MP T cells resulting in enhanced homing to the gut cannot be excluded, and could explain a lower contribution of Hoxb4 CD4 memory T cells to the lymphoid organs.

In addition, it is still plausible that CD4 MP T cells might respond differently to Hoxb4 than HSCs. It might be that homeostatic proliferation of MP T cells is predominantly governed by cytokine signalling, while intrinsic signalling pathways play a larger role in HSCs.

In conclusion we show that Hoxb4 favours the maintenance and expansion of CD4 central MP cells following acute homeostatic proliferation, which suggest a more robust preservation of CD4 MP T cells in the long-term.

Supporting Information

Figure S1 Analysis of T cell populations in young and old Hoxb4 transgenic mice. Graphs showing the average size of CD4 (A) and CD8 (B) populations in lymphoid organs of young (2–3 months of age) and old (>15 months of age) Hoxb4 transgenic (n=7) and wt (n=7) age matched mice. *P<0.05, 2-tailed Student t test. WT = wild type, LN = Lymph node; BM = bone marrow.

Figure S2 Absolute CD4 MP T cell numbers following homeostatic proliferation. (A) Absolute number of Hoxb4 and wt CD4 MP T cells in lymphoid organs of primary hosts after 2 months of competitive proliferation. The calculations of the absolute numbers are based on 8 LNs, Spl and BM derived from 2 legs. Data are obtained from 9 mice in 3 independent experiments. *P=0.03; 2-tailed Student t test. (B) Absolute number of Hoxb4 and wt CD62L positive CD4 MP T cells in primary hosts (n=6). The numbers of CD62L MP T cells in the initial graft are calculated based on percentage of CD44hi/CD62L+ population as given in Table 1. Note the expansion of the CD62L+ population in several mice. *P=0.01; 2-tailed Student t test. WT = wild type, MP = memory phenotype, LN = Lymph node, Spl = spleen, BM = bone marrow.

Figure S3 Production of cytokines after stimulation with PMA/ionomycin. Percentage of Hoxb4 and wt MP T cells (gated on CD44hi) in secondary and tertiary hosts positive for indicated cytokines (n=3–6). WT = wild type, MP = memory phenotype, TNF = tumor necrosis factor; IL-2 = interleukine-2; IFN = interferon.

Table S1 Average cell numbers (×106) in hematopoietic organs of Hoxb4 and wt mice.

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Author Contributions

Conceived and designed the experiments: NL, JB.Performed the experiments: HG, PT. Analyzed the data: HG, PT. NL, JB.Contributed reagents/materials/analysis tools: NL, JB. Wrote the paper: NL, JB. Mounting of figures: MF, HG, JB.
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