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Expansion of the CD8 memory T cells: implications for the self-renewal gene *Hoxb4*

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Résumé

Les cellules CD8 T mémoire (Tm) offrent une protection tout au long de la vie contre les infections récurrentes. Elles sont maintenues grâce à des mécanismes d'auto-renouvellement. Les cellules souches hématopoïétiques (CSH) peuvent aussi s'auto-renouveller lentement, en assurant leur maintenance à long terme. Les deux types de cellules utilisent la moelle osseuse comme principale niche de prolifération. CD8 Tm et CSH partagent partiellement un profil transcriptionnel, y compris certains gènes connus pour contrôler l'auto-renouvellement. Les gènes *Hox*, dont *Hoxb4* qui est un activateur puissant de l'expansion des CSH *in vitro* et *in vivo*, sont exprimés par les CSH. Basé sur les similitudes entre les CSH et les cellules Tm, nous émettons **l'hypothèse** que les gènes impliqués dans l'auto-renouvellement des CSH, comme *Hoxb4*, favoriseront l'expansion des cellules CD8 Tm. Pour tester cette hypothèse, nous avons déterminé l'effet de la surexpression de *Hoxb4* dans les cellules T à partir de souris transgéniques jeunes et âgées, et sur la prise de greffe et le maintien des cellules CD8 Tm après leur transplantation dans des souris immunocompétentes ou immunosupprimées.

À l'état basal, la composition et le phénotype des cellules T naïves et mémoire n'ont pas été affectés par la surexpression de *Hoxb4*. Pour étudier le maintien des cellules CD8 Tm, les souris transgéniques *Hoxb4* et OT-1 ont été croisées. Les cellules T des souris OT-1, entre autre, expriment un récepteur de lymphocyte T qui reconnaît le peptide d'ovalbumine (OVA), présenté par le complexe majeur d'histocompatibilité de classe I. En utilisant ce modèle, nous avons généré *in vitro* un grand nombre de cellules CD8 Tm pour les expériences du transfert adoptif. Ces cellules sont monoclonales et spécifiques pour la reconnaissance d'OVA. Le nombre et le phénotype des cellules CD8 Tm générées en culture n'ont pas été affectés par la surexpression de *Hoxb4*. Ces cellules ont été transplantées dans des souris de type sauvage afin d'évaluer leur prise de greffe et leur maintien à long terme. Après la transplantation chez des souris soit immunocompétentes ou immunosupprimées, la prise de greffe et le maintien des cellules CD8 Tm surexprimant *Hoxb4* n'a pas été améliorée lorsque comparée aux cellules CD8 Tm de type sauvage. Ces résultats suggèrent que *Hoxb4* ne favorise pas l'auto-renouvellement des cellules CD8 Tm. D'autres études vont essayer d'identifier les facteurs contrôlant l'auto-renouvellement des cellules CD8 Tm.

Mots-clés: *Hoxb4*, cellules CD8 T mémoire, cellules souches hématopoïétiques, auto-renouvellement, prolifération homéostatique et modèles de souris transgéniques.

Abstract

CD8 memory T (Tm) cells provide life long protection against recurrent infections and are maintained through self-renewal mechanisms. Hematopoietic stem cells (HSC) also possess the capacity to slowly self-renew assuring their long-term maintenance. Both cell types use the bone marrow as their preferred proliferation niche. CD8 Tm and HSC partially share a transcriptional profile including some genes known to control self-renewal. *Hox* genes are expressed in HSCs, among them *Hoxb4* is a potent enhancer of HSC expansion *in vitro* and *in vivo*. Based on the similarities between HSC and Tm cells, we **hypothesize** that genes involved in HSC self-renewal, like *Hoxb4*, will promote CD8 Tm cell expansion. To test this hypothesis, we have determined the effect of *Hoxb4* overexpression in T cells from young and old transgenic mice, and on the engraftment and maintenance of CD8 Tm cells after transplantation into immunocompetent or immunodeficient mice.

At the steady-state, the composition and phenotype of the naïve and memory T cells were not affected by *Hoxb4* overexpression. To study CD8 Tm cell maintenance, the *Hoxb4* and the OT-1 transgenic mice were crossed; the latter express a T cell receptor that recognizes the ovalbumin (OVA) peptide, presented by major histocompatibility complex class I. Using this model, we generated *in vitro* large numbers of monoclonal and OVA specific CD8 Tm cells for adoptive transfer experiments. The number and the phenotype of Tm cells generated in culture were not affected by *Hoxb4* overexpression. These cells were transplanted into wild type mice to evaluate their engraftment and long-term maintenance. After transplantation in either immunocompetent or immunodeficient mice, *Hoxb4* overexpressing Tm cell engraftment or maintenance was not enhanced when compare to wild-type Tm cells. These results demonstrate that *Hoxb4* does not promote CD8 Tm cell self-renewal. Further studies will try to identify the factors controlling CD8 Tm cell self-renewal.

Keywords: *Hoxb4*, CD8 memory T cells, hematopoietic stem cells, self-renewal, homeostatic proliferation and transgenic mice model.

Table of contents

List of tables

List of figures

Abbreviations

Abd: abdominal

Ag: antigen

AML: acute myeloid leukemia

Antp: antennapedia

APC: antigen presenting cells

Baso: basophil

BM: bone marrow

BMP: bone morphogenetic protein

CAR cells: CXCL12-abundant reticular cells

CFSE: carboxyfluorescein succinimidyl ester

CLP: common lymphoid progenitor

CMP: common myeloid progenitor

CXCL12: CXC-chemokine ligand 12

DC: dendritic cell

Dfd: Deformed

E: embryonic days

Eomes: Eomesodermin

Eosin: eosinophil

FACS: Fluorescence Activated Cell Sorting

GATA2: Gata binding protein 2

Gfi1: growth factor independence 1 gene

GMP: granulocyte-macrophage progenitor

Hox: homeobox

HP: lymphopenia induced homeostatic proliferation

HSC: Hematopoietic stem cell

IFN-I: type I interferon

IL: Interleukin

KLRG1: killer cell lectin-like receptor G1

Lab: labial

Lck: lymphocyte-specific protein tyrosine kinase

LMPP: lymphoid-primed multipotent progenitor

LN: lymph node

Macro: macrophage

Meg: megakaryocyte,

MHC: major histocompatibility complex

MkPE: megakaryocyte-erythrocyte progenitor

MLN: mesenteric lymph nodes

Mono: monocyte

MPP: multipotent progenitor

MP T: memory-like T cell phenotype

Nk: natural-killer

Neutro: neutrophil

OVA: ovalbumin

Pb: proboscipedia

Pc: peritoneal cavity

RBC: red blood cell (erythrocyte)

Runx1: Runt-related transcription factor 1 gene

Sca-1: stem cell antigen-1

Scr: Sex combs reduced

SDF-1: stromal cell derived factor 1

SDLN: skin draining lymph nodes

Shh: Sonic hedgehog

SPL: spleen

STAT3: signal transducer and activator of transcription 3

T-ALL: T-cell acute lymphoblastic leukemia

Tcf-1: T cell factor 1

Tcm: central memory T

TCR: T cell receptor

T_{DIM}: death intermediates memory T cells

Te: effector T

Tem: effector memory T

Th: T helper

Tm: memory T

Tn: naïve T

TNF: tumor necrosis factor

TRAF: TNF receptor-associated factors

Trm: tissue-resident memory T

Tscm: memory stem T cells

Ubx: Ultrabithorax

Wnt: Wingless-type

WT: wild type

Acknowledgements

Introduction

1.- Hematopoietic stem cells

One of the most extremely regenerative tissues in the human body is the blood. It is very complex, but at the same time, it is probably one of the most studied and understood developmental systems. Its organization consists of cellular hierarchies where all the cells come from a common precursor which continues to differentiate to give rise to all of mature blood cell types. The hematopoietic stem cells (HSCs) are at the base of this hierarchy. They self-renew and differentiate to maintain the production of the blood cells. The self-renewal is a cellular division which is critical to preserve the multipotential differentiation capacity of the parental HSC in at least 1 of the daughter cells (Sieburg et al., 2013).The principal niche of HSCs is the bone marrow (BM), where they receive the necessary signals for their function (Doulatov et al., 2012).

The HSCs qualities (self-renewal and differentiation potential) can be proven when the cells can fully reconstitute lethally irradiated mice after transplantation. This has been the leading assay to the discovery and understanding the HSCs. From the 60's it was realized that colony-forming-units could generate similar colonies upon transplantation into secondary recipients although it was difficult to purify the HSC (Lessard et al., 2004). By the end of the 80's, different phenotypic markers were used to purify and characterized the HSCs. Spangrude and collaborators (Spangrude et al., 1988) showed that 50% of lethally irradiated mice are rescued with 30 cells (Lin$^-$ Thy-1^{low} Sca-1$^+$). Then c-kit was showed to be essential for hematopoiesis in BM and self-renewal of hematopoietic progenitor cells (Ogawa et al., 1991). These experiences result in the LSK (Lineage$^{-/low}$, Sca-1$^+$, c-Kit$^+$) marker set use canonically to identify HSCs enrichment. The identification of the SLAM family receptors (including CD150, CD244, CD229 and CD48) has allowed a better detection of HSCs from the LSK cells fraction in the BM (Kiel et al., 2005; Oguro et al., 2013). Currently, transplantation of a single HSC into lethally irradiated mouse result in long-term reconstitution of the lymphohematopoietic system (Osawa et al., 1996).

The majority of the HSCs are in a quiescent state dividing infrequently, but they can cycle with regularity renewing the pool in few weeks. In order to respond to hematopoietic stress like BM injury, HSCs can switch from the quiescent to the active state increasing their division for replenishing the HSC pool or differentiating to replenish the missing mature blood cells. When normal levels of cells are regained, activated HCSs return to the quiescent state (Fleming et al., 1993; Wilson et al., 2008).

1.1.- Ontogeny

During embryonic development, the niches for the HSCs and blood cell production change. Mouse primitive hematopoiesis begins at embryonic day (E) 7.5 in the yolk sac, where primitive erythroid cells are produced. These cells will function to deliver oxygen to the embryo and they are the predominant blood cell type before the definitive erythrocyte production. Then, around E8.25, myelo-erythroid progenitor cells from yolk sac will migrate to the fetal liver where they will generate enucleated erythrocytes. These cells will rapidly become more numerous than the primitive population (Baron et al., 2013; Clements and Traver, 2013; Frame et al., 2013; Sugiyama et al., 2011). At E8.5 the appearance of the first multipotent progenitors in the yolk sac and the placenta has been demonstrated (Alvarez-Silva et al., 2003). At E12.5, definitive hematopoiesis starts in the fetal liver, where HSCs have arrived and expanded. Then, they can enter the fetal spleen (SPL) at E13.5. Finally, after E16.5 the adult hematopoiesis is established with the migration of HSCs to the fetal bone marrow where they will stay to generate the different blood cell types (Baron et al., 2013; Clements and Traver, 2013; Sugiyama et al., 2011). At the end of gestation, when BM becomes the dominant site, the number of HSC is stabilized and this number will be maintained in adult life. It is not clear where definitive HSCs are generated during embryogenesis before liver formation at E11.5. The different origins proposed are the extraembryonic mesoderm at the yolk sac, the vascular endothelium in the intraembryonic para-aortic-splanchnopleural mesoderm / aorta-gonad-mesonephros region and also the proposition that some cells come from the placenta (Adamo and Garcia-Cardena, 2012; Medvinsky et al., 2011; Sugiyama et al., 2011).

Adult hematopoiesis is a process of cell differentiation and proliferation resulting in the production of all the different blood cell types (**Figure 1**). The understanding of this hierarchical process has changed from a simple linear differentiation to a system that is much more complex. In a simplified way, HSCs will progressively differentiate generating multipotent progenitor cells with an intermediate or short-term repopulation capacity. These progenitor cells gradually continue to acquire characteristics of a more specific lineage. Then, they will generate one of the lineage committed cells like the megakaryocyte-erythrocyte progenitor (MkEP), granulocyte-macrophage progenitor (GMP) and the common lymphoid progenitor (CLP). In turn, the lineage committed cells will finish their differentiation into specific mature blood cell types. Intrinsic and extrinsic factors are involved in defining the decision of the cellular fate (Lensch, 2012; Orkin, 2000; Rieger and Schroeder, 2012).
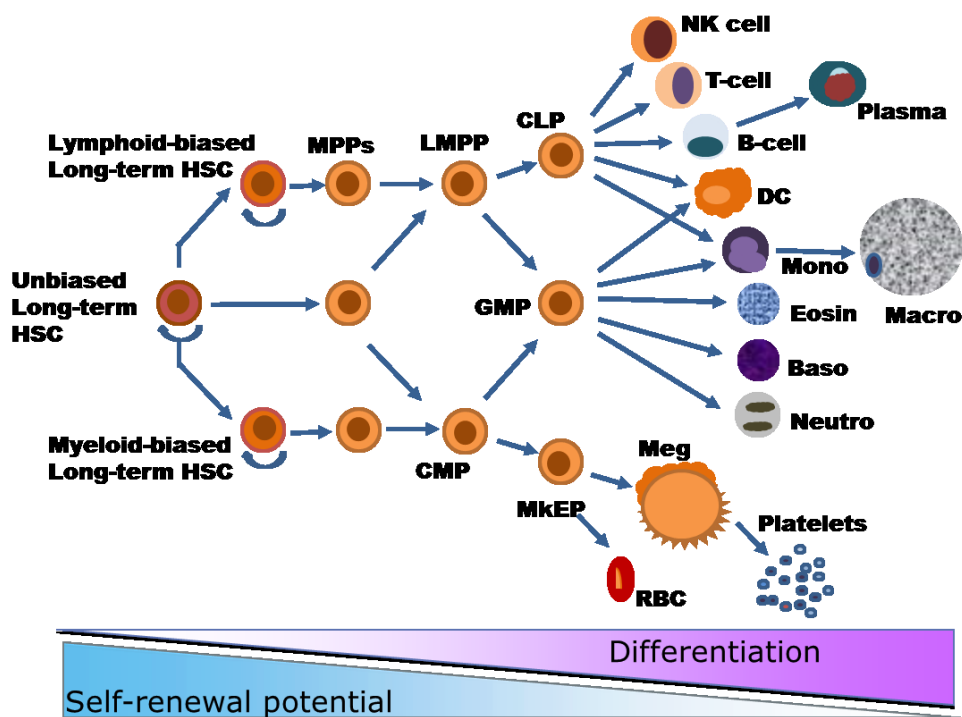
Figure 1.- Blood cell ontogeny.
Hematopoietic stem cells can self-renew and differentiate to produce all the mature blood cell types. The self-renewal potential is lost with the progressive differentiation. HSC = Hematopoietic stem cells, MPP = multipotent progenitor, CMP = common myeloid progenitor, LMPP = lymphoid-primed multipotent progenitor, MkEP = megakaryocyte-erythrocyte

4

progenitor, GMP = granulocyte-macrophage progenitor, CLP = common lymphoid progenitor, RBC = red blood cell (erythrocyte), Meg = megakaryocyte, Neutro = neutrophil, Baso = basophil, Eosin = eosinophil, Mono = monocyte, Macro = macrophage, DC = dendritic cell, NK cell = natural-killer cell. Adapted from: An Evolving Model of Hematopoietic Stem Cell Functional Identity. *Stem cell reviews* (Lensch, 2012).

Originally, HSCs were considered as a single homogenous population of cells, however recent studies showed that HSCs are heterogeneous and have different regeneration potentials. Three types of HSCs have been proposed based on their ability to generate different lineage (**Figure 1**). These are the unbiased that divide into all, the myeloid-biased and the lymphoid-biased long term HSCs (Babovic and Eaves, 2014; Lensch, 2012; Zhang et al., 2013).

1.2.- Hematopoietic stem cell niches

HSCs need to be maintained in the body without losing their ability to differentiate and to self-renew. HSCs are located in a specific microenvironment, the "HSC niche" which supplies the necessary components and signals to maintain and to regulate the cycling state of HSCs (Isern and Mendez-Ferrer, 2011). The principal niche of adult HSCs is located in the BM (Doulatov et al., 2012). Within the BM, two specific microenvironments are identified: the endosteum niche and the vascular niche.

1.2.1.- The endosteum niche

The inner surface between the bone and the BM is known as the endosteum. This surface is well vascularized. HSC populations and primitive hematopoietic progenitors are localized in the endosteum. Different mechanisms have been suggested for the way the endosteal cells contribute to the maintenance of HSCs. These cells can be acting by direct contact, releasing soluble factors or regulating the function of intermediate cells (Chotinantakul and Leeanansaksiri, 2012; Kiel and Morrison, 2008).

The endosteal cells can produce some of the necessary factors for the HSC maintenance, however these factors are still not completely characterized. The osteoblasts localized in this surface are suggested to be the main supportive cells for HSCs, because they provide factors that can regulate the maintenance, quiescence and localization in the niche of HSCs, including soluble factor as angiopoietin-1, stromal cell derived factor 1 (SDF-1) and osteopoietin (Chotinantakul and Leeanansaksiri, 2012; Kiel and Morrison, 2008).

Besides the role to support HSCs maintenance in normal conditions, the endosteum seems to play an important role when the BM is damaged, promoting HSC expansion. For example, irradiation can damage the BM and eliminate HSCs from the body. When HSCs are transplanted into irradiated mice, they preferentially localize in the endosteum where they expand to re-establish the blood cell populations of the mice. The irradiated niche produces cytokines. This localization is different when HSCs are transplanted to normal mice where they are randomly distributed (Xie et al., 2009).

1.2.2.- The vascular niche

The vascular niche is also thought to be involved in the maintenance of the HSCs. During the development of the embryo, vascular or perivascular niches are probably the primary residence for HSCs before they finally migrate to the BM through the vascular system (Kiel and Morrison, 2008). The vascular system also plays a role in adult HSCs migration given that the cells can be mobilized rapidly into circulation after stimulus like interleukin-8 (IL-8) (Laterveer et al., 1995).

Within the vascular niche, the endothelial cells are suggested to be the providers of the necessary signals for the maintenance and differentiation of the HSCs (Chotinantakul and Leeanansaksiri, 2012). However, it is still not clear if the endothelial cells are needed for both mechanisms or just for one (Kiel and Morrison, 2008). Endothelial signaling seems to be

mediated through the cytokine receptors that share the subunit gp130. Transplantation assays demonstrated that when HSCs are transplanted into mice with endothelial cells deleted for gp130, normal hematopoiesis is not re-established and mice fail to recover, even if HSCs enter the BM (Yao et al., 2005). These results suggest that endothelial cells are important for the maintenance of hematopoiesis; however, it is still not clear if the endothelial cells are acting directly or indirectly on HSCs (Kiel and Morrison, 2008).

1.2.3.- Additional cell types supporting HSCs

Other cells, like the reticular cells, could have a role contributing to the niche for HSCs either directly or indirectly. Probably, the HSCs niche can be considered a combination of all the different supporting cells (Kiel and Morrison, 2008).

An example of these cells is the reticular cells known as CAR cells, which reside in the perivascular niche. This name was given because they abundantly produce CXC-chemokine ligand 12 (CXCL12) also known as SDF-1. HSCs are in contact with CAR cells. CXCR4 is the primary receptor for CXCL12. When this receptor is deleted in HSCs, their number is reduced. In contrast, this deletion does not affect more mature progenitors. It seems that the signaling trough CXCR4 provided by CAR cells is important for the maintenance of HSCs (Sugiyama et al., 2006).

Every day, numbers of HSCs are entering and exiting the circulation, which means that HSCs are not only staying in the BM. In the mouse, between 100 to 400 HSCs are estimated to be circulating in the blood (Wright et al., 2001). This opens the possibility that HSCs could have other niches that are still not defined. Other cell types and microenvironments could be supporting the maintenance of HSCs by providing direct or indirect signals. Moreover, after injury, the mechanisms that maintain HSCs could change to induce the appropriate response of the cells (Kiel and Morrison, 2008).

1.3.- Self-renewal of HSCs

To prevent exhaustion of HSCs that would jeopardize blood cell production, HSCs divide without differentiation to maintain numerical stability, a process also called self-renewal. Depending on the requirement of the organism, HSCs will self-renew to maintain or to expand the population. To obtain either maintenance or expansion of HSCs, self-renewal distinguishes either symmetrical or asymmetrical division (Lessard et al., 2004).

The symmetrical division is defined as a division resulting in two daughter cells that preserve the characteristics of the parental cell. It is also known as self-renewal expansion. It is observed during embryogenic development when HSCs expand in the fetal liver. In adult, hematopoiesis an expansion of HSCs only occurs when hematopoietic stress takes place, for example following transplantation or BM injury. The mechanisms controlling HSCs self-renewal in both, embryonic and stress hematopoiesis, may be similar (Lacadie and Zon, 2011; Lessard et al., 2004).

The asymmetrical division results in two different daughter cells. One will preserve the characteristics of the parental cell and the other will be more differentiated and continue the path to produce the blood cells necessaries for the body (Ting et al., 2012). It has been observed that the asymmetrical division of HSCs can produce one daughter that remains quiescent while the other multiplies (Huang et al., 1999). The molecular mechanism is still not well known. With the use of videomicroscopy it was observed that the endocytic protein AP2A2 is asymmetrically segregated during mitosis of HSCs, suggesting that this protein could play a role in this type of division (Ting et al., 2012). In contrast to the segregation during asymmetric division of this protein, it was shown that chromosomes are randomly segregated (Kiel et al., 2007).

To maintain the HSCs pool and to give rise to the different blood cell types a balance between symmetrical and asymmetrical division is required (Huang et al., 1999). Studies are ongoing to unravel the mechanisms regulating the self-renewal of the HSC.

1.4.- Molecules involved in HSC self-renewal

There are multiple factors known to be involved in the regulation of HSC self-renewal. Very generally, they can be divided in intrinsic or extrinsic factors. The intrinsic control of HSC self-renewal includes transcription factors and chromatin-associated factors regulating the gene expression, like the homeobox (*Hox*) genes and the growth factor independence 1 gene (Gfi1). The extrinsic factors coming from the environment could modulate the gene expression. There are multiple extrinsic regulators of HSCs self-renewal like growth factors, cytokines or chemical modulators. HSCs express receptors for different extrinsic regulators. These receptors can activate developmental pathways that could modulate the fate of HSCs. Wingless-type (Wnt), Notch and Sonic hedgehog (Shh) are among these pathways (Walasek et al., 2012).

HSCs express Gfi1 which seems to play a role in the homeostasis of the cells. Gfi1 function to restrain HSC proliferation. When these cells are Gfi1 deficient, they highly proliferate which leads to cellular exhaustion and reduced population in the host BM. These deficient cells are also incapable of sustaining hematopoiesis and mice reconstitution after transplantation (Hock et al., 2004; Zeng et al., 2004). The regulation of this gene is very complex; it seems to be controlled by 5 different transcription factors including Runt-related transcription factor 1 gene (Runx1) and the Gata binding protein 2 (Gata2) (Wilson et al., 2010). In adult HSCs, Runx1 deletion inhibit the production of the lymphocyte progenitors (Growney et al., 2005), whereas Gata2 expression induces a quiescent state in HSCs (Tipping et al., 2009). Moreover, Gata2 is implicated in survival and proliferation of HSCs (Tsai and Orkin, 1997). Both roles seem to be agreeing with the Gfi1 role to restrain HSCs excessive proliferation (Wilson et al., 2010).

The Wnt is a main pathway in HSCs self-renewal. The Wnt signaling pathway is involved in the regulation of the HSCs fate. During hematopoiesis, Wnt is activated and regulates HSCs fate in a dose dependent manner. β-catenin is the canonical signaling transducer of this pathway leading to the activation of gene transcription. (Luis et al., 2011; Walasek et al., 2012). The inhibition of Wnt pathway by blocking its receptor (Frizzled) leads to inhibition of

cellular growth *in vitro* and reduce *in vivo* reconstitution of HSCs (Reya et al., 2003). On the other hand, the overexpression of β-catenin can expand the HSCs pool *in vitro*. Wnt activation can induce the expression of other genes involved in HSCs self-renewal, like *Hoxb4* and Notch1. The different regulators involved in the self-renewal of HSC could interact in a hierarchical way. However, during HSC self-renewal, Wnt signaling could have different outcomes depending on the levels of activation which resulted in expansion at intermediates levels and exhaustion at higher levels (Luis et al., 2011). Moreover, Wnt signalling has also been related to ageing, with a reduction in the self-renewal capacity of the HSCs (Florian et al., 2013). The elevated expression of Wnt5a seems to induce HSC ageing, as it induces this process in young HSCs and its haploinsufficiency reduces HSCs ageing. The different outcomes produce by Wnt signaling could be the result of a complex regulation of this pathway.

During hematopoiesis, the Notch pathway plays a role modulating the balance between lineage specificity and self-renewal of HSCs. Constitutive activation of the Notch1 receptor leads to an increase in HSCs numbers due to enhance self-renewal and decrease differentiation. It also favors lymphoid over myeloid lineage formation (Stier et al., 2002). However, this pathway is not essential for hematopoiesis because mice with Notch1 and one of its ligand Jagged1 inactivation can normally survive, and HSCs with Notch1 deficiency can reconstitute irradiated normal or a Jagged1 deficient host after transplantation (Mancini et al., 2005).

The protein, Sonic hedgehog is also involved in HSCs self-renewal. The pathway that it activates can lead to HSC expansion. Shh signaling acts through downstream bone morphogenetic protein (BMP) signaling (Bhardwaj et al., 2001). While BMP-4 can increase the survival of HSCs, the inhibition of BMP-4 can block the expansion induced by Shh signaling. Interestingly, a link placing the Wnt canonical signaling through β-catenin downstream of Shh signaling has been made in human myeloid leukemia progenitors (Su et al., 2012).

As discussed above, different regulators are involved in HSCs self-renewal and could be participating together. Shh seems to be place upstream of Wnt which can induce Notch1. The *Hox* genes also play an important role in HSCs hematopoiesis and self-renewal that will be further discussed. The interactions of different mechanisms controlling the self-renewal of HSCs

10

are very complex. Dysregulation of this balance could lead to diverse hematopoietic malignancies.

2.- *Hox* genes

Hox genes are characterized by a homeodomain which consist of 183 base pairs coding a helix-loop-helix that allows binding to the DNA (Gehring and Hiromi, 1986; Hill et al., 1989). These genes are known as master transcription factors, they are at the top of the cascade developmental programs and they control the expression of multiple genes. *Hox* genes control the body plan of the embryo, are highly regulated and have been highly conserved throughout evolution. They were discovered and characterized in *Drosophila* by their ability to regulate the position identity along the anterior-posterior body axis during development. The *Hox* genes play key functions in early hematopoietic cells (Alharbi et al., 2013; Argiropoulos and Humphries, 2007; Gehring et al., 1994). Besides, it was shown that, in order for *Hox* genes to bind its DNA target, they require the action of cofactors (Popperl et al., 1995).

The *Hox* genes consist of 39 genes in mammals. They are located in 4 chromosomes and divided into 4 clusters known as A, B, C and D (**Figure 2**). Comparing the composition of these clusters between species, paralogue groups are observed. These paralogues groups are very similar in sequence and redundant in function. There are 13 paralogue groups in mammals. (Alharbi et al., 2013; Argiropoulos and Humphries, 2007; Krumlauf, 1994).

This study will focus on the *Hoxb4* gene. *Hoxb4* is a member of the *HoxB* cluster localized on mouse chromosome 11. It is expressed during development and in adult human and mouse hematopoietic stem and progenitor cells. Interestingly, it is down regulated with the differentiation of HSCs (Pineault et al., 2002; Ramirez-Solis et al., 1993).
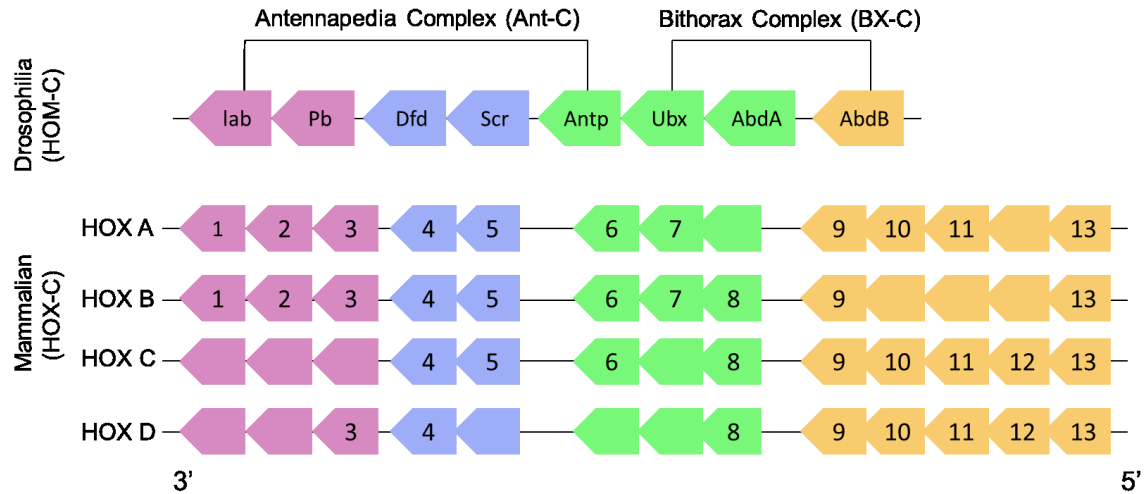
Figure 2.- Schematic representation of *Hox* genes clusters.
The four *Hox* clusters (A, B, C and D) contain 8-11 genes. The identical colors represent the paralog groups. Human and Drosophila homologies are also shown by colors. Adapted from: The role of *Hox* genes in normal hematopoiesis and acute leukemia, *Leukemia*; and *Hox* genes in hematopoiesis and leukemogenesis, *Oncogene* (Alharbi et al., 2013; Argiropoulos and Humphries, 2007). Lab = labial, Pb = proboscipedia, Dfd = Deformed, Scr = Sex combs reduced, Antp = antennapedia, Ubx = Ultrabithorax, Abd = abdominal (A or B).

2.1.- *Hox* genes in hematopoiesis

In the hematopoietic cells, the *Hox* genes undergo down-regulation of expression with differentiation and some of them like the *HOXB3* and *HOXB4* are almost exclusively expressed in the most primitive BM cells (Pineault et al., 2002; Sauvageau et al., 1994). Another example is the *HoxA* cluster. This cluster is express at higher level in HSCs compared to early progenitors and when its expression is diminished, the maintenance of HSCs is also reduced (Lebert-Ghali et al., 2010).

However, it has been difficult to determine the precise role of the *Hox* genes in hematopoiesis. Single *Hox* genes mutants cannot always help to determine their role. A reason for this difficulty is that the *Hox* genes share overlapping function in addition to unique roles. For example, during embryonic development *Hoxa3* and *Hoxd3* can play the same biological role and they present particular roles when they are quantitatively modulated (Greer et al., 2000).

The role of *Hoxb4* in hematopoiesis has been studied extensively. Studies on the *Hoxb4* mutant showed that this gene is not necessary for the generation of HSCs during embryogenesis or the definitive hematopoiesis because the animals lacking this gene can normally develop although they have minor alterations (Bijl et al., 2006; Brun et al., 2004; Ramirez-Solis et al., 1993). It has been shown with the double *Hoxb3*/*Hoxb4*-deficient mice that HSCs lacking these genes have impaired repopulation capacities and slower cell cycle, demonstrating that both genes are required for the maximal proliferative response (Bjornsson et al., 2003). Moreover, under stress hematopoiesis, like when the HSCs are transplanted into irradiated mice, the capacity of *Hoxb4* deficient HSCs to expand and repopulate the niche is reduced (Brun et al., 2004). Indicating that *Hoxb4* plays a role in the enhancement of the HSCs self-renewal capacity.

Retroviral overexpression studies in mouse showed that *Hoxb4* is a potent enhancer of HSC expansion *in vivo* and *in vitro*, without leading to leukemia (Sauvageau et al., 1995). After BM transplantation, the numbers of HSCs are not recovered to normal levels but cells overexpressing *Hoxb4* can fully reconstitute the HSC compartment (Thorsteinsdottir et al., 1999). Moreover, the activation of the Wnt pathway leading to an enhancement of HSC self-renewal is associated with increased *Hoxb4* expression suggesting that the expansion of HSCs by Wnt is mediated through *Hoxb4* (Reya et al., 2003).

The function of *Hoxb4* is compensated by the up regulation of genes from other cluster genes, because depletion of *Hoxb1-b9* did not affect HSC functional reconstitution of mouse (Bijl et al., 2006). Therefore, other *Hox* genes could provide similar expansion on HSCs. In the case of *Hoxa4* overexpression, the expansion of the cells is unbalanced resulting in higher preferences for lymphoid over myeloid progenitors (Fournier et al., 2012). *Hoxc4* overexpression leads to the expansion of human CD34$^+$ hematopoietic cells (Auvray et al., 2012).

The *Hox* genes are linked to the self-renewal program crucial for HSCs. However, high *Hox* gene expression have been observed in myeloid and lymphoid leukemias, which could be providing the self-renewal capacity to the cancer stem cells (Argiropoulos and Humphries, 2007). The cancer stem cells are able to regenerate the tumor, causing relapse and metastasis. Properties associated to HSCs are also found in cancer stem cells, they possess the ability to self-renew and differentiate into all the cell types that are specifics for the particular tumor or cancer. It has been suggested that signaling pathways controlling the self-renewal are common to both cancer and hematopoietic stem cells (Lobo et al., 2007).

For acute myeloid leukemia (AML) a poor prognosis has been associated with an increased expression of homeodomain transcription factors including *Hoxb3, Hoxb4, Hoxa7-11*. The overexpression of Hox proteins leads to the expansion of different BM populations. It can cause the block of the differentiation and the generation of myeloproliferation and AML *in vivo* (Eklund, 2011). *Hoxa9* is an example, its overexpression is associated with a very poor leukemic prognosis and it can promote immortalization of HSCs (Brumatti et al., 2013). The homeodomain region of this gene can intrinsically control the transformation from a normal to a leukemic cell. When this homeodomain region is exchanged with the same region in *HOXA1*, it can induce the aggressive features observed in *HOXA9*; while realising the opposite experiment leads to a reduction of the aggressiveness (Breitinger et al., 2012).

In T-cell acute lymphoblastic leukemia (T-ALL), chromosome rearrangements lead to the overexpression of oncogenic transcription factors, including *Hox* genes. Proteins that control developmental checkpoints in early thymocytes are collaborating with these genes that control HSCs self-renewal. The consequence is the increased self-renewal and survival of the abnormal thymocytes (Tremblay et al., 2010). The childhood T-ALL presents another interesting characteristic, the up regulation of β-catenin expression in more than 85% of the patients. When this gene is silenced, the cancerous cells become more apoptotic (Ng et al., 2014). As discussed before, the Wnt-β-catenin pathway plays an important role in HSCs self-renewal and it can induce other genes like *Hoxb4* (Luis et al., 2011; Reya et al., 2003; Walasek et al., 2012).

2.2.- Modulation of the *Hoxb4* effect in HSCs self-renewal

Besides the compensatory effect of the paralog *Hox* genes, different molecules could be involved in the modulation of *Hoxb4* effect in HSCs self-renewal like the signal transducer and activator of transcription 3 (STAT3) and the Co-factors like Pbx1.

The activation of STAT3 can lead to HSCs expansion in a similar way to the expansion induced by *Hoxb4* overexpression. However, activating both does not produce an additive effect. In contrast, when Stat3 is inhibited, it can reduce the effect caused by *Hoxb4* overexpression. Moreover, *Hoxb4* and STAT3 present an overlap in the transcription factors induced in HSCs, including pluripotent genes involved in the maintenance of cellular stemness. Therefore, it seems that in HSCs self-renewal regulation, the effect of *Hoxb4* passes through STAT3 and that both factors can play an equivalent role inducing similar outcomes (Hong et al., 2014).

Pbx1 is a homeoprotein of the TALE subfamily that function as a cofactor for Hox proteins modulating their binding to DNA (Chang et al., 1995). It has been shown that reduction of *Pbx1* expression, using a retroviral construct with antisense *Pbx1*, the effect of *Hoxb4* overexpression is enhanced. Therefore, *Pbx1* could be a negative regulator in this process. Most likely, multiple cofactors act in combination to modulate the *Hoxb4* induction of self-renewal in HSCs (Cellot et al., 2007).

As discussed, *Hoxb4* can induce HSCs self-renewal without surpassing a normal expansion of the cells. However, the mechanisms controlling HSCs self-renewal are very complex involving several molecules. The interactions of the different mechanisms need to be well controlled to avoid the development of malignancies.

3.- Adaptive immunity

To defend the body, innate and adaptive (acquired) immune mechanisms are used. The innate immunity, activated by chemical properties of the antigen (Ag), consists of nonspecific defense like physical barriers, chemicals and cells that attack foreign cells. The adaptive immunity consists of an Ag specific response; in this case, the Ag is processed and recognized, then the cells that are capable of recognition will clear the infection. Very importantly, the adaptive immunity creates memory cells that will be maintained in the body in order to provide a more efficient defence against a subsequent encounter with the specific Ag.

When pathogens like virus and bacteria reinvade the body, the memory cells from the adaptive immunity are activated in order to mount a stronger and faster attack on the recurrent infection. The key for this ability is the immunological memory capable of recognized the antigens expressed by the recurrent invaders (Smith, 2012). The B and T lymphocytes that can recognize and respond to practically any peptide non-self Ag are the basis of this system (Janeway, 2001b). Large repertoires of B and T cell receptors (TCR) are randomly generated, creating a library for the recognition of peptide non-self Ag.

3.1.- Lymphocytes

The lymphocytes are the central players of the adaptive immune system. As all the types of mature blood cells, they differentiate from the HSCs that go through a series of sequential divisions generating intermediates known as precursors, in a process that will end with the formation of particular lineages (Karsunky et al., 2008). The existence of a lymphoid-biased long term HSC (**Figure 1**) that differentiate to generate all T cells, B cells and NK cells has been proposed (Lensch, 2012).

3.1.1.- T Lymphocytes

To become a T cell, lymphocyte precursor cells migrate periodically from the BM and enter the thymus. (Saran et al., 2010; Schwarz and Bhandoola, 2006; Yang et al., 2010). The thymus has a distinctive anatomical organization that allow the entering cells to move in microenvironments perceiving signals that are critical for the development of T cells. Functional T cells capable of recognize non-self Ag but also to be tolerant to self Ag exit the thymus (Starr et al., 2003). These mature cells are CD4 or CD8 T cells called naïve (Tn) because they have not encountered the Ag that they are capable of recognizing through their TCR (Ma et al., 2013; Starr et al., 2003). These mature T cells recirculate through the secondary lymphoid organs in order to encounter their specific Ag which is brought to these organs by the Ag presenting cells (APCs).

The TCR is a disulfide-linked heterodimer of α and β chains. There is a TCR composed of γδ chains, which is less abundant than the αβ TCR but structurally similar. The Ag binding site is compose by 6 complementary determining regions formed by each chain, been 3 α and 3 β chains. A variable and a constant Ig-like domain are the composition of each chain. The variable region is the one that binds the Ag-MHC complex. The constant domain is followed by the transmembrane region and a short cytoplasmic tail. The TCR is expressed at the membrane in a complex with the CD3 chains which are responsible for the signal transmission. The co-receptors CD8 or CD4, via their cytoplasmic domain, are associated with the TCR-CD3 complex. The TCR binds the membrane distal Ag-MHC molecule while simultaneously, the co-receptors CD8/CD4 bind distant to this site and nearest to the membrane, to the invariant domain of the Ag-MHC helping with the process of cell activation (Bhati et al., 2014; Huang et al., 2012; Janeway, 2001a).

The TCR recognize the peptide Ag only when presented by the MHC molecules on the own cells surface, but not the Ag or the MHC alone. For the recognition, TCR requires the help of co-receptors (Huang et al., 2012). The CD8 T cells can recognize the Ag presented by the MHC class I, while the CD4 T cells recognize the Ag presented by the MHC class II (Sprent et

al., 2008). The absence of the co-receptor signal impairs the differentiation and activation of the T cells. The co-receptor expressed by each T cell is associated to the action of the T cell. The T cells expressing CD4 are regulatory/helper/inducer T cells while the T cells expressing CD8 are killer/cytotoxic T cells (Mak, 1994).

3.1.2.- T cell activation

The Ag recognition by the TCR can lead to the activation of T cells. The activation of T cells requires three important signals: **1)** the recognition of the Ag by the engagement of the TCR to MHC molecules presenting the antigenic peptide, **2)** a co-stimulation signal provided by the interaction of the CD28 receptor on the T cells with the costimulatory ligands on the APCs and **3)** cytokine stimulation (Mescher et al., 2007).

3.1.2.1.- First signal: TCR engagement

The recognition of the Ag peptide on the MHC by the TCR is the beginning of the T cell signaling. However, the TCR does not have signaling domains and in order to generate a signal, it is coupled with the CD3 complex. The CD3 complex consists of four accessory chains (two ε, one δ, one γ) and the ζ chain, which is a large intracytoplasmic homodimer. This complex is associated with two αβTCR heterodimers in the cell surface (Castro et al., 2014; Janeway, 2001a; Mak, 1994; Smith-Garvin et al., 2009). After TCR engagement with Ag peptide-MHC, the CD3 complex initiates a signaling cascade activating the cells. The CD3 complex has immunoreceptor tyrosine-based activation motifs (ITAMS) that become phosphorylated and recruit other molecules to initiate the signaling cascade (Birnbaum et al., 2014; Guy and Vignali, 2009; Huang et al., 2012; Smith-Garvin et al., 2009). The co-receptors CD4 or CD8, upon association with the MHC, are responsible for the ITAMS phosphorylation required for the T cell activation The lymphocyte-specific protein tyrosine kinase (Lck), a Src family kinase, is associated to the

cytoplasmic domains of the co-receptors and phosphorylates the ITAMs of the CD3 complex (Castro et al., 2014; Huang et al., 2012; Janeway, 2001a; Mak, 1994; Smith-Garvin et al., 2009).

3.1.2.2.- Second signal: T cell co-stimulation

For Tn cells, the first signal is not sufficient to induce a complete T cell response. Therefore, other cell surface receptors play an important role in providing the signals needed for activating T cells. T cell anergy (long-lived non-responsive state), deletion or development of immune tolerance are produced when T cell activation occurs without co-stimulation. The CD28 receptor expressed by T cells can robustly provide the co-stimulation signal. It can interact with the co-stimulatory ligands B7-1 (CD80) and B7-2 (CD86) that are present on the surface of activated APCs. The result of this engagement is an increase in the magnitude and the regulation of the response generated by the first signal. On the other hand when this signal is not provided, the ability to respond to some pathogens can be dramatically reduced (Mak, 1994; Mescher et al., 2007; Smith-Garvin et al., 2009).

3.1.2.3.- Third signal: Cytokine stimulation of T cells

Now that the T cells have interacted with the APCs, recognized and respond to the Ag, a third signal is required for Tn cells in order to differentiate, expand and survive. It consists of inflammatory cytokines that can be produced by different cells including the APCs. For CD8 T cells, the cytokines IL-12 and type I interferon (INF-I) have been largely studied for their role, leading to full activation, proliferation and memory generation. The cytokine requirement was evident when CD8 T cells were purified and *in vitro* stimulated with artificial APCs. CD8 T cells were capable of receiving the first and second signal, but they were not able to fully proliferate; however, the addition of the cytokines IL-12 or INF-I leads to a stronger proliferation of the cells. The lack of these cytokines leads to reduction in the cell ability to respond and when both

receptors are eliminated, there is no memory generation (Curtsinger et al., 2003; Curtsinger et al., 1999; Curtsinger et al., 2005; Keppler and Aichele, 2011; Mescher et al., 2007). For CD4 T cells, the third signal is very important for making the decision to differentiate into a specific effector type. Common gamma c cytokines are involved in this decision. The common gamma chain is a cytokine receptor sub-unit common for different interleukin receptors including IL-2, IL-4, IL-7, IL-9, IL-15, IL-21. For example: IL-12 and INF-γ are involved in the differentiation of helper T 1 cells, IL-4 and IL-2 in helper T 2 cells, transforming growth factor β and IL-6 in helper T 17 cells, IL-6 and IL21 in follicular helper T cells, IL-2 and transforming growth factor β in regulatory T cells (Dong, 2008; Luckheeram et al., 2012; Yamane and Paul, 2013).

3.2.- CD8 T cell response

Remembering the encountered pathogen leads to enhanced responses after a secondary encounter. The bases for this immunological memory are specialized cells known as memory T (Tm) and B cells. This mechanism has been used to generate a successful vaccination, inducing immunological long-lived protection against specific Ag. (Kaech et al., 2002b).

The journey to generate CD8 Tm cells begins with the CD8 Tn cells exiting the thymus, entering the secondary lymphoid organs including the lymph nodes (LN) in order to scan APCs looking for their specific Ag (Munoz et al., 2014). Notably, LN structure facilitates the contact of CD8 T cells with APCs, providing an important niche for the antigen surveillance and the generation of the immune response (Mandl et al., 2012; Munoz et al., 2014). APCs migrate to the secondary lymphoid organs like the draining lymph nodes after taking the Ag in the infection place (Janeway, 2001a).

During the normal course of an immune response, CD8 Tn cells will encounter a foreign specific Ag that they can recognize and get activated. Three phases of T cell response occur following this activation. The **expansion** is the first one, the activated Tn cells will expand and differentiate into effector T (Te) cells. CD8 Te cells can release cytokines like INF-γ which

directly inhibits viral replication, they can kill target cells that display the specific peptide fragments on MHC-I at the cell surface by secreting perforin and granzymes, and they can also trigger apoptosis in some target cells trough Fas ligand. With this, they eliminate the pathogen within days. The second phase, known as the **contraction** period, follows the Ag clearance. The majority of the CD8 Te cells die during this period (>90%). The **memory** phase is the third phase, a few (<10%) CD8 Te cells further differentiate into CD8 Tm cells that will be maintained for life through self-renewal mechanisms (Badovinac et al., 2002; Cui and Kaech, 2010; Harty and Badovinac, 2008; Joshi and Kaech, 2008).

3.2.1.- CD8 Tm cell generation

Following Ag clearance, different CD8 Tm cell populations are generated, although it is still not clear how this process works . Different models of CD8 Tm cell differentiation have been suggested, for example: A) the Tn cells can give rise to Te or directly to Tm cells. B) The Te cells will generate Tm cells after Ag clearance (Kaech et al., 2002b).

Given the heterogeneity of the CD8 Te cells, certain characteristics have been used to try to distinguish which cells will generate CD8 Tm cells. At the peak of the immune response, cells that are proposed to be the memory cells precursors (killer cell lectin-like receptor G1 (KLRG1) $^{lo/int}$ CD127^{hi}) are present (Gerlach et al., 2011). KLRG1 cells appear during early stages of infection. The CD8 T cells expressing hi KLRG1 go downregulating IL-7 receptor and become the short-lived effector cells. While the cells with low levels of KLRG1 keep high expression of IL-7 receptor and become the memory precursor effector cells. During the priming of CD8 T cells, inflammatory molecules like IL-2 can instruct the signaling process leading to the choose between memory precursor or short-lived effector cells. IL-2 can dose dependently modulates T-bet. In turn, high levels of T-bet induce high expression of KLRG1 and low levels of T-bet induce low expression of KLRG1 (Joshi et al., 2007). It has been suggested that the acquisition of the memory properties is a progression of events giving rise to the different Te cell populations. During the immune response, Te cells receive different signals like Ag and inflammatory molecules. Differences in the duration and the quantity of these signals, during the

different stages of the immune response, can influence the cells decisions between death after Ag clearance or survival and differentiation into Tm cells (Gerlach et al., 2011). Changes in the gene expression between Tn, Te and Tm cells support the notion of progressive differentiation of naïve T cells into Tm cells (Kaech et al., 2002a; Sarkar et al., 2008).

3.3.- Main differences between Tn and Tm cells

The Tm cells, that are generated after Tn cells encounter for the first time their specific Ag and mount an immune response, possess some characteristics that allow them to better respond the next time the Ag is encountered (Joshi and Kaech, 2008; Kaech et al., 2002b).

First, there are few (tens to hundreds of cells per mouse) Tn cells capable of recognizing one specific pathogen. In contrast, the numbers of Ag specific Tm cells exceed the number of Ag specific Tn cells. That is because during the immune response, each Tn cells will divide generating more than 10,000 cells, then less than 10% of these number of cells will survive after the contraction period as Tm cells. This means that there are more cells ready to eliminate the pathogen for the next infection (Harty and Badovinac, 2008).

Second, while Tn cells recirculate through secondary lymphoid organs, Tm cells can also migrate to peripheral sites where pathogens generally enter the body. As Tm cells are present in the sites of infections, this facilitates the encounter with the pathogen (Ebert et al., 2005; Weninger et al., 2001).

Third, for the activation of the CD8 T cells, in contrast with Tn cells, the CD8 Tm cells do not required co-stimulation (Flynn and Mullbacher, 1996).

Fourth, CD8 Tm cells also have an increased sensitivity to Ag compared to Tn cells because they have redistribute their TCR resulting in enriched oligomeric TCR complexes (Kumar et al., 2011). Also, during the immune response, the Tn cells that possess a higher affinity to bind Ag-MHC complexes will selectively respond expanding and generating the Tm

cells. Resulting in CD8 Tm cell population expressing TCRs with higher affinity for the Ag-MHC complexes (Busch and Pamer, 1999).

Fifth, compared to Tn cells, Tm cells divided after a shorter time interval and present different effector functions in the same cells (Veiga-Fernandes et al., 2000).

Sixth, CD8 Tm cells are more ready to respond than CD8 Tn cells because they retain some characteristics of CD8 Te cells like the expression of several cytotoxic proteins, chemokines and cytokines. Moreover, comparing their gene expression patterns, CD8 Tm cells are between CD8 Tn and CD8 Te cells, thus sharing some characteristics with both cell types leading to a more rapid response (Goldrath et al., 2004; Holmes et al., 2005; Kaech et al., 2002a; Veiga-Fernandes and Rocha, 2004).

Seventh, with age Tn cell number diminish. On contrary, Tm cells survive for long period providing long-term protection. For example, people vaccinated against smallpox retain the ability to respond after 75 years (Hammarlund et al., 2003; Vescovini et al., 2014).

In summary, the increased number of highly sensitive Ag-specific Tm cells, their anatomical location, their faster division, non-requirement for co-stimulation and their gene expression patterns that facilitates their faster responses, together with their longevity explains why Tm cells provide an enhanced long-term protective immunity compared to Tn cells.

3.4.- CD8 Tm cell populations

The different CD8 Tm cell populations that have been describe include central memory T (Tcm) cells, effector memory T (Tem) cells, memory stem T cells (Tscm), lymphopenia induced homeostatic proliferation (HP) Tm cells and tissue-resident memory T (Trm) cells. Some of the characteristics of these cells are shown in Table 1.

3.4.1.- Characteristics of the different CD8 Tm cell populations

The CD8 Tcm cells are preferentially located in lymphoid tissues, and they can strongly proliferate in response to Ag encounter. CD62L and CCR7, which allow efficient homing to secondary lymphoid tissue, are expressed on the surface of these cells and have been used to distinguish them from other Tm populations. CD8 Tcm can better persist *in vivo* and as they possess an increased proliferation potential, they are more efficient to mediate immunity compare to the Tem cell (Huster et al., 2004; Weninger et al., 2001; Wherry et al., 2003). This was demonstrated by evaluating the ability to rapidly control pathogen growth after transferring the CD8 Tcm or Tem cells in different viral challenge models (Wherry et al., 2003).

The CD8 Tem cells recirculate through blood and spleen and migrate to non-lymphoid tissues. As Tcm, they possess the ability to generate an immediate effector function after Ag encounter. CD8 Tem cells barely express CD62L or CCR7 or not at all (Masopust et al., 2001). IL-7 receptor (CD127) is expressed on their surface as well as on Tcm cells (Huster et al., 2004).

The CD8 Tscm population was identified studying graft-versus-host disease. They are capable of maintaining some characteristics of a Tn phenotype like low expression of the hyaluronan receptor CD44 and high levels of CD62L; in contrast, they express higher levels of stem cell antigen-1 (Sca-1), CD122 and Bcl-2. Tscm are capable of enhanced expansion after Ag-reencounter and it seems that this population is also capable of generating both Tcm and Tem populations (Yu and Anasetti, 2005; Zhang et al., 2005). Gattinoni and collaborators have characterized a human T cell population with the features of Tscm. This cell population can self-renew and differentiate into Tcm, Tem and Te cells (Gattinoni et al., 2011; Gattinoni et al., 2009). Moreover, Cieri and collaborators have found that Tscm accumulate when Il-7 and IL-15 availability is high, their gene expression profile is between Tn and Tcm cells and Tscm can mediate the graft versus host disease (Cieri et al., 2013).

The lymphopenia induced HP Tm cells were identified studding different models like after transferring CD8 Tn cells to T cell depleted host, neonatal models and Ag-free mice

(Haluszczak et al., 2009; Hamilton and Jameson, 2008; Hamilton et al., 2006; Kieper and Jameson, 1999; Le Campion et al., 2002; Sprent and Surh, 2011). These cells do not express early activation markers (CD69, CD25, CD71) but they progressively express memory markers (CD44, CD122, Ly6C) with division (Murali-Krishna and Ahmed, 2000). They also acquire the abilities to lyse target cells and secrete INF-γ. (Cho et al., 2000; Dummer et al., 2002). For CD8 HP Tm cells to acquire these characteristics, CD8 Tn cells need to divide multiple times (Cho et al., 2000). It was proposed that CD8 HP Tm cells can protect the host without interfering with the CD8 Tm cell populations that are normally generated during infection. The CD8 HP Tm cell population can respond to infection but when they are challenged in competition with CD8 Tm cells, CD8 HP Tm cells are less effective and die more quickly (Cheung et al., 2009).

CD8 Trm cells are a distinguishable population of CD8 Tm cells that is established in nonlymphoid organs. In humans, Sathaliyawala and collaborators have characterized the Trm cell populations in lymphoid and mucosal tissues from individual donors. These cells express the activation marker CD69, CD103 which allow them to adhere to E-cadherin and is linked to their homing into epithelial tissues, and they are capable of INF-γ and IL-2 production after stimulation while the other memory T cell populations do not express CD69 nor CD103 (Sathaliyawala et al., 2013). Another Trm cell population was identified in the murine submandibular glands, which also express CD69 and CD103. Interestingly, after adoptive transfer of these murine Trm cells and rechallenge, they preferentially relocalized into their original tissue (Hofmann and Pircher, 2011). In intestinal epithelium, Trm have been studied by Masopust and collaborators. They found that the Trm cell population did not migrate and rather stay in their tissue. They use two approaches, one was evaluating the migration of Trm cells in mice after transplanting part of the intestine containing Trm cells, the other was evaluating the number of Trm cells after treating mice with FTY720 which induce the accumulation of recirculating lymphocytes into the lymphoid tissue while reducing their presence in other tissues (Masopust et al., 2010). Schenkel and collaborators have proposed that Trm cells serve as local sensor capable of recruiting circulating Tm cells. In the case of female mouse reproductive tract, after rechallenge, they found that CD8 Trm cells can potentiate local response and recruitment of CD8 Tm circulating cells; they also observed the expression of INF-γ by these cells (Schenkel et al., 2013). In general, we can say that the CD8 Trm cells have distinctive characteristics from

Table 1.- Characteristics of mouse CD8 T cell populations.

		CD8 T cells					
		Naive	**Effector**	**Memory stem**	**Central memory**	**Effector memory**	**Resident memory**
Surface phenotype	CD44	L	H	L	H	H	
	CD62L	H	L	H	H	L	
	Ly6C	L/M	L/M	H	H	H	N/L
	1B11	L	H		L	H	
	CD127	M/H	N/L	M/H	H	M/H	N/L
	Sca-1	N/L	M	H	M/H	M	
	CD69	N	H	M/H	N	N	H
	CD28	L/M	H	H	H	N /L	
	CCR7	H	N/L	H	H	N/L	N
	CD122	N/L	M/H	H	M/H	M/H	
	CD25	L	H	L	L	L	N
	Bcl-2	M/H	L	H	H	L/M	
	KLRG1	N/L	H	N	L	L/M	N
	CD103	N	N/L	N	N	N/L	H
Effector function	IFNγ	N	H	N/L	M	H	H
	TNFα	N	H/M		H	H/M	H
	IL-2	N	L/M	L	H	N/L	H
	Cytoxicity	N	H	N/L	L	H	H
	Self-renewal	L		H	M/H	L	M/H
Survival Factors		IL-7		IL-7	IL-7	IL-15	
				IL-15	IL-15		
Transcription Factors	EOMES	L	L/M	L	L	M	L
	TCF7	H	L/M	H	M	L	
	FOXP1	H	N/L	M	L	N	

Adapted from: Paths to stemness: building the ultimate antitumor T cell, *Nat. Rev. Cancer*; Anti-viral CD8 T cells and the cytokines that they love, *Virology*; Memory stem cells sustain disease, Nat. Med.(Cox et al., 2013; Gattinoni et al., 2012; Yu and Anasetti, 2005). N = negative, L = low, M = medium, H = high.

other CD8 Tm cell populations, among them, their no recirculation and preferential localization to their specific nonlymphoid organs where they function to locally generate an immune response.

3.4.2.- CD8 Tm cell populations differentiation models

Different models have been proposed to describe the differentiation of the CD8 Tm cell populations. The linear model proposes that CD8 Tn cells differentiate into Te cells and then into Tm cells; after this, the differentiation from Tcm to Tem or the inverse have been proposed. Another model proposed that Tcm and Tem differentiate directly from effector cells (Gerlach et al., 2011; Lefrancois and Marzo, 2006).

Wherry and collaborators support the linear model: Tn $\rightarrow$ Te $\rightarrow$ Tem $\rightarrow$ Tcm differentiation pathway. Using adoptive transfer of Tcm or Tem cell population into naïve host, they show that Tem convert into Tcm in the absence of Ag. They consider the Tcm cells as the "true memory" with the ability to self-renew and to survive long-term. When CD8 Tcm cells are re-exposed to Ag, they rapidly proliferate and generate effector response (Wherry et al., 2003).

The other model suggests that both Tcm and Tem are separate terminal lineages with different capacity to self-renew and home (Lefrancois and Marzo, 2006). Evidence coming from human studies supports this idea, since no conversion between Tcm and Tem was found when following the circulating human repertoire over 9 months (Baron et al., 2003). Another evidence was found by Marzo and collaborators, they did not find conversion from Tem to Tcm or vice versa by studying polyclonal populations of CD62L $^{high\ or\ low}$ CD8 Tm cells separately transferred to naïve mice (Marzo et al., 2005).

3.4.3.- CD8 Tm cell populations generation

For CD8 Tcm cells, the Wnt pathway seems to be important for the generation of these cells. The T cell factor 1 (Tcf1) is a nuclear effector of this pathway. Mice lacking Tcf-1 present a normal primary response generating Te and Tem cells, but they do not respond well to a secondary challenge as the generated cells expand poorly and they lack CD8 Tcm cells (Jeannet et al., 2010). On the other hand, constitutive activation of Wnt signaling promotes Tcm generation and higher response in a secondary challenge (Zhao et al., 2010).

For CD8 Tem IL-2 seems to play a role in their generation. When CD8 T cells are primed in culture in the presence of IL-2, the resulting cells are similar to CD8 Tem cells and they accumulate in inflamed tissues but not in lymphoid organs (Weninger et al., 2001). CD8 T cells lacking the receptor for IL-2 poorly generated Tem cells and CD8 Tm cells lacking the receptor for IL-2, after secondary challenge, cannot repopulate the effector compartment (Mitchell et al., 2010).

For the generation of CD8 Tscm, IL-7, IL-15 and the CD3/CD28 engagement seems to be important (Cieri et al., 2013). Very interestingly, Wnt signaling can promote the generation of these cells suggesting a role for this pathway on the maintenance of the stem cell-like characteristics for the mature CD8 Tm cells (Gattinoni et al., 2011; Gattinoni et al., 2009).

In the case of the CD8 HP Tm cells, it is proposed that the free space and the contact with self-MHC ligands promote the changes in the Tn cells that with subsequent divisions, will lead to the generation of the HP Tm cells (Goldrath and Bevan, 1999; Hamilton and Jameson, 2008; Kieper and Jameson, 1999). Compared with CD8 Tm cells, the CD8 HP Tm cells do not required IL-2 or co-stimulation via CD28 for their generation (Cho et al., 2000). Moreover, while cytokines like IL-4 or IL-15 enhance the generation of CD8 HP Tm cells, IL-7 is critical for their generation (Tan et al., 2001).

The generation of CD8 Trm cells is not well understood. It seems to be related to the site of the infection, where they will preferentially stay (Sheridan et al., 2014). In mucosal tissues, inhibition of the mammalian target of rapamycin blocks the CD8 Trm formation (Sowell et al., 2014).

3.5.- Homeostatic maintenance of T cells

The immune system needs to maintain Tn cells to be capable of recognizing all the possible invaders. To assure the defense of the host from reinvading pathogens, the immune system needs to maintain the memory cells. The niche provide the factors needed for their maintenance and the T cell numbers are stably maintained over time (Nolz et al., 2012). Homeostatic maintenance mechanisms work to preserve a varied functional pool of T lymphocytes that will survive a long time (Boyman et al., 2012). Homeostatic proliferation mechanisms promote the cell proliferation in conditions where for example the numbers are reduced by irradiation or drugs, in order to re-establish the cell levels to normal numbers; this process is known as lymphopenia induced proliferation. The factors involved in homeostatic proliferation and survival vary within the different T cell populations, although some mechanisms are common (Boyman et al., 2009).

CD8 Tm cells are present in both lymphoid (LN, SPL, BM) and nonlymphoid tissues (peripheral tissues like lung or skin). Their preferred site for proliferation is the BM: CD8 Tm cells divide more in the BM compare to other locations (Becker et al., 2005; Parretta et al., 2008). In the case of CD4 Tm cells, it has been observed that during the following weeks after their generation, these cells migrate to the BM where they are maintained in a resting and highly reactive state (Tokoyoda et al., 2009). Thus, the BM works as the niche for both HSCs and Tm cells providing them with the necessary signals for their maintenance.

Different survival factors must be provided by the niche to maintain T cell homeostasis. For example, in the LN, the T zone fibroblastic reticular cells are the principal source of IL-7

which has been involved in T cell survival (Link et al., 2007). In the secondary lymphoid organs, the stromal cell networks are responsible for providing homeostatic factors and guidance to the trafficking of T cells (Takada and Jameson, 2009a).

3.5.1.- Factors involved in Tn cell homeostasis

The interaction of the TCR with self-peptide-MHC-I complexes is required for homeostatic maintenance (survival) of CD8 Tn cells (Ernst et al., 1999; Takada and Jameson, 2009b). Besides, after CD8 or CD4 Tn cells are respectively transferred to MHC-I or MHC-II depleted and irradiated mice, the proliferation of these cells is minimal (Tan et al., 2002).

Different cytokines are involved in the homeostatic maintenance and proliferation of CD8 Tn cells. Studies *in vitro* have found that IL-4, IL-7 and IL-15 play a role in the survival of CD8 Tn cells and enhance homeostatic proliferation *in vivo* (Boise et al., 1995; Tan et al., 2001; Vella et al., 1997). For example, mice lacking IL-15 present reduced Tn populations (Kennedy et al., 2000). However, only IL-7 was found to be a critical factor for both, the survival and homeostatic proliferation *in vivo*, because CD8 Tn cells hardly proliferate when transferred to hosts lacking this cytokine and they disappear with time (Tan et al., 2001). Additionally, CD4 Tn cell proliferation is not enhanced by the addition of IL-4, IL-7, IL-15 or their combination *in vitro*. CD4 Tn cells, as CD8 Tn cells, do not proliferate in IL-7 deficient mice even if the mice have more available resources due to the lack of mature T and B cells, like in the $RAG^{-/-}$ mice (Schluns et al., 2000). In the case of human CD4 Tn cells isolated from umbilical cord blood, they can be maintained in culture by the addition of IL-7 and they modestly proliferate (Webb et al., 1999).

CD8 Tn cells die when they receive prolonged IL-7 signaling if they do not possess TCR low-affinity for self-peptide MHC-I (Kimura et al., 2013). To allow the homeostasis of CD8 Tn cells, a mechanism of coreceptor tuning was proposed. It is the result from the interplay between the IL-7 cytokine and the TCR signaling (Park et al., 2007). On one hand, IL-7 signaling induces

more CD8 expression, which in turn promotes TCR engagement to self-ligands. On the other hand, TCR signaling impairs cytokine signaling. The reduction in the cytokine signaling leads to a decrease in the expression of CD8. When CD8 is less express, TCR engagement is reduce allowing the effect of IL-7 cytokine signaling and re-starting the cycle. Thus, the CD8 Tn cell homeostatic maintenance is dependent on IL-7 and on signals received from the TCR engagement of self-peptide MHC complexes (Takada and Jameson, 2009a).

3.5.2.- Factors involved in Tm cell homeostasis

Different factors have been studied for their relation with Tm cells homeostasis. Including cytokines, receptors for the tumor necrosis factor (TNF), the Wnt signaling pathway and factors involved in Tn cell homeostasis like the TCR engagement with self-peptide-MHC-I.

3.5.2.1.- The role of the interaction TCR-self peptide in Tm cell homeostasis

While Tn cells require an interaction with self MHC molecules for survival, Ag specific CD8 Tm cells do not need TCR engagement to survive, because they can survive and divide after transferring them into MHC-I deficient mice (Murali-Krishna et al., 1999). T cells from a transgenic mouse model which possesses the particularity that tetracycline administration blocks TCR expression, were transferred into normal mice and followed after immunization to observe the generated Tm cell populations (Leignadier et al., 2008). When TCR expression is ablated at the Tm stage by tetracycline administration, the CD8 Tm cells continue to normally self-renew and survive. Besides, studying the cells in a model where the transgenic TCR expression is affecting only 10 to 20% of CD4 T cells, following the CD4 Tm phenotype cells after the tetracycline administration, it was showed that a subset of these cells were capable of surviving

even in competition with the normal cells (Leignadier et al., 2008). Furthermore, when CD8 or CD4 Tm phenotype cells are transfer to MHC-I or MHC-II (respectively), or MHC-I-II deficient mice which were rendered lymphopenic by irradiation, both cell types extensively proliferate while Tn cells minimally proliferate (Tan et al., 2002). Therefore, TCR engagement is not required for CD8 or CD4 Tm cell homeostatic proliferation during lymphopenia, self-renewal or survival as it is required for CD4 and CD8 Tn cells.

3.5.2.2.- The role of cytokines in Tm cell homeostasis

Transferring OT-1 CD8 Tm cells into IL-7$^{-/-}$ irradiated mice, showed that there was a reduction in the homeostatic proliferation of these cells compared to the transfer into non-irradiated or irradiated normal mice (Schluns et al., 2000). Indicating that IL-7 is required for the proliferation of a subset of CD8 Tm cells under these conditions. When CD8 Tm cells are transferred into lymphopenic IL-15 deficient hosts, the cells can survive but they hardly divide. On the other hand, CD8 Tm cells can slowly proliferate when transplanted into lymphopenic IL-7 deficient mice. But when the lymphopenic IL-15 deficient mice are also treated with anti-IL-7Rα, the homeostatic proliferation of CD8 Tm cells is completely blocked (Goldrath et al., 2002). In the case of CD4 Tm phenotype cells sorted from normal non-immunized mice, their homeostatic proliferation is not affected when transplanted into lymphopenic IL-7 or IL-15 deficient mice, neither when transplanted into lymphopenic IL-15 deficient mice treated with anti-IL-7/7Rα (Tan et al., 2002). In contrast, after immunization, sorted CD4 Tm cells that are transplanted into irradiated normal, IL-7, IL-15 or IL-7/15 deficient mice indicated that they are more dependent on IL-7 for their proliferation and only when both cytokines are missing their proliferation is abolished (Purton et al., 2007). Thus, IL-7 and IL-15 have no role in homeostatic proliferation during lymphopenia in CD4 Tm phenotype cells. While CD4 Tm cells that are generated after immunization are more dependent on IL-7 and like CD8 Tm cells they fail to proliferate only when both IL-7 and IL-15 signals are missing under lymphopenic conditions. CD8 Tm cells can use either IL-7 or IL-15 to perform homeostatic proliferation during lymphopenia (Goldrath et al., 2002; Purton et al., 2007; Tan et al., 2002).

CD8 Tm cells divide slowly under homeostatic conditions. They are absent in IL-15R deficient mice and their proliferation is reduced when this receptor is blocked by antibodies (Ku et al., 2000). The role of IL-15 and IL-7 in homeostatic proliferation was also studied in full lymphoid compartments. The CD8 Tm cells transferred into normal hosts are able to self-renew, but when transfer into IL-15 deficient hosts they hardly divide. When the CD8 Tm cells are transferred into normal hots treated with anti-IL-7Rα, their proliferation is slightly reduced compared to non-treated mice. Therefore, IL-7 is not necessary while IL-15 is essential for their self-renewal homeostatic proliferation in a full compartment (Goldrath et al., 2002). Experiments transferring Ag experienced CD4 Tm cells, non-Ag experienced CD4 Tm phenotype cells and CD8 Tm phenotype cells into normal hosts which then received IL-15 injection, showed that the CD8 Tm phenotype and the Ag experienced CD4 Tm cells proliferate in response of IL-15, while the CD4 Tm phenotype cells were indifferent. Transferring the Ag experienced CD4 Tm cells into IL-15 or IL-7 deficient host showed a little division that is eliminated in the IL-7/15 deficient host, showing the need of Ag-experienced CD4 Tm cells for both cytokines in order to self-renew in a full compartment (Purton et al., 2007).

Studies with thymectomized mice treated with anti-IL-7Ra, showed a decay in the total numbers of CD4 and CD8 cell populations suggesting a role for this cytokine in the survival of the cells (Vivien et al., 2001). Comparing OT-1 and OT-1 IL-7R$^{-/-}$ cells transferred to normal mice, which were then infected with vesicular stomatitis virus expressing ovalbumin and monitored for the T cell response and memory production, it was found that the memory population consist almost only of OT-1 cells sufficient for IL-7R. Showing that without IL-7 the CD8 Tm cells cannot survive (Schluns et al., 2000). Experiments performed in full lymphoid compartments also lead to a better understanding of the role of IL-15 and IL-7 in the survival of the cells. CD8 Tm cells can be found in IL-15 deficient hosts 50 days after transferring the cells, presenting a little reduction in their number compare to the cells transferred to normal hosts. However, anti-IL-7Rα treatment of normal or IL-15 deficient hosts leads to a major decrease in the numbers of CD8 Tm cells recovered with time (Goldrath et al., 2002). Therefore, IL-7 is necessary for the survival of CD8 Tm cells in a full compartment, while IL-15 is not critical. In the case of Ag experienced CD4 Tm cells, when they are transplanted into IL-7, IL7R deficient

or anti-IL-7 treated mice, there is a progressive loss of the cells, in addition the CD4 Tm phenotype cells from the mice treated with anti-IL-7 are also decline, suggesting the IL-7 dependence of memory phenotype and Ag experienced memory CD4 T cells for their survival (Kondrack et al., 2003). Moreover, Ag experienced CD4 Tm cells transferred into IL-15 deficient mice showed a reduction in the number of recovered cells with time, compared to transplantation into normal mice. This reduction is even greater when the cells are transplanted into IL-7 or IL-7/15 deficient mice, showing a bigger requirement of IL-7 compared to IL-15 for the survival of Ag experienced CD4 Tm cells in a full compartment (Purton et al., 2007).

3.5.2.3.- The role of TNF family ligands in Tm cell homeostasis

In addition to cytokines, signaling trough the receptors for the TNF family ligands, including OX40, CD30 and 4-1BB, has an impact on Tm cell maintenance. After infection, not only numbers of CD8 Tm cells generated in 4-1BB deficient mice are reduced with time compared to normal mice, but also the secondary response is affected, suggesting its involvement in the survival and turnover of Tm cells (Bertram et al., 2002). Furthermore, 4-1BBL (Ligand)$^{-/-}$ mice generates a normal primary immune response, but with lower numbers of Ag-experienced CD8 Tm cells and are less capable of eliminate the infection after challenge compared to normal mice, supporting the involvement of 4-1BB in the survival of CD8 Tm cells (Tan et al., 2000). CD8 Tm cells survive less when transferred into 4-1BBL deficient hosts. Moreover, IL-15 can induce the *in vitro* expression of 4-1BB in a dose and time dependent manner on CD8 Tm cells (Pulle et al., 2006). Therefore, Sabbagh and collaborators proposed a model suggesting that after encountering a source of IL-15, cells upregulate 4-1BB activating this signaling pathway, leading to upregulation of anti-apoptotic molecules and downregulation of pro-apoptotic molecules, and finally resulting in an increased cell survival (Sabbagh et al., 2007). The TNF receptor-associated factors (TRAFs) are a family of adapter proteins that mediate the signal of the pro-survival members of the TNFR family, connecting them with downstream signaling pathways like c-Jun N-terminal kinase, p38 mitogen-activated protein kinases and nuclear factor kappa-light-chain-enhancer of activated B cells pathways. Mice with a

dominant negative form of TRAF2 have T cell defects and dramatically impaired secondary T cell response (Cannons et al., 2002). Moreover, TRAF1 deficient CD8 Tm cells accumulate higher levels of pro-apoptotic molecules *in vitro* compared to normal cells, and present a decreased recovery *in vivo* (Sabbagh et al., 2006), providing more evidence for the role of the TNF family in the survival of CD8 Tm cells.

The OT-1 Ox40$^{-/-}$Cd30$^{-/-}$ CD8 Tm cells were compared to OT-1 CD8 Tm cells, both generated after transferring spleen cells into Rag-deficient hosts in competition. The OT-1 Ox40$^{-/-}$Cd30$^{-/-}$ CD8 Tm cells were less detectable after 14 days post-primary immunization and also after 63 days post-secondary immunization, indicating their role in the survival of CD8 Tm cells (Bekiaris et al., 2009). Besides, using OT-1 with single deficiency, Ox40$^{-/-}$ was observed to present a more dominant effect compared to Cd30$^{-/-}$, but their consequence was synergistic, showing their contribution in the survival of CD8 Tm cells. For CD4 Tm cells, the lamina propria of the intestine is a major reservoir. CD30 or OX40 deficiency reduces the total CD4 T cell numbers in the small intestinal lamina propria, and the depletion of the cells is severe when both are absent. Transferred OT-II Ox40$^{-/-}$Cd30$^{-/-}$ CD4 T cells into normal mice fail to persist 7 days after immunization, compared to OT-II CD4 T cells transferred in competition, showing that they are required for the survival of CD4 T cells in the lamina propria (Withers et al., 2009). This was also observed by transferring OT-II normal and Ox40$^{-/-}$Cd30$^{-/-}$ CD4 T cells in competition into RAG-deficient mice, 3 weeks after immunization the CD4 Tm deficient cells where found markedly reduced compared to the normal OT-II CD4 Tm cells (Gaspal et al., 2005). Therefore, the TNF family members are associated to the survival of both, CD4 and CD8 Tm cells after immunization.

3.5.2.4.- The role of Wnt signaling pathway in Tm cell homeostasis

The Wnt signaling pathway plays a role in CD8 Tm cell generation and has also been suggested to play a role in CD8 Tm cell homeostasis (Zhou et al., 2010). The nuclear effector of this pathway is Tcf-1. Compared to normal cells transferred in competition, CD8 Tm cells

deficient in TCF-1 have a significantly diminished secondary proliferation after transferring into normal hosts and re-challenge. Moreover, TCF-1 deficient CD8 Tm cells are progressively lost with time after transferring into normal hosts and present a diminished BrDU uptake, which demonstrates defective self-renewal. Moreover, CD8 Tm cells deficient in TCF-1 present a reduced proliferation *in vitro* in the presence of IL-15, which is an important factor that stimulates the self-renewal proliferation. IL-2Rβ also known as CD122, is a shared receptor subunit between IL-2 and IL-15 and its expression was found to be decreased in TCF-1 deficient CD8 Tm cells. Eomesodermin (Eomes), a paralogue of T-bet, was also found among the most downregulated genes in TCF-1 deficient CD8 Tm cells by transcriptome analysis. Eomes$^{+/-}$ mice had a decrease in CD8 Tm phenotype cell populations which is more notorious in Eomes$^{+/-}$T-bet$^{-/-}$ mice (Intlekofer et al., 2005). Comparing Eomes KO mice with normal mice, their CD8 central memory phenotype T cells are less abundant. In mice chimeras compose of Eomes KO and normal BM, 30 and 70 days after infection, a reduction in the CD8 Tm cell population generated from Eomes KO was observed compared to the CD8 Tm cells generated from normal mice. Besides, BrdU incorporation 60 days after infection was reduced in the Eomes KO CD8 Tm cells in BM, suggesting a defect in the self-renewal of these cells. As in Eomes$^{+/-}$T-bet$^{-/-}$ mice and the negative induction of Eomes *in vitro*, Eomes KO CD8 Tm cells possess a modest defect in CD122 expression. Eomes KO CD8 Tm cells also present a reduced expression of Bcl-2 mRNA, proposing a survival disadvantage (Banerjee et al., 2010). These experiments suggest that Eomes could be involved in the self-renewal and the survival of CD8 Tm cells. In the case of CD4 Tm cells, larger numbers of Ag-specific cells are generated under constitutive activation of the Wnt pathway (Zhao et al., 2010), and T-bet has also been found higher expressed in human CD4 T helper (Th) 1 Tem cells and Eomes in CD4 Th1 Tem and Tcm cells (Knox et al., 2014). The CD4 Th1 cell population derived from CD4 Th17 cells possess the ability to long-live and self-renew as memory or stem cells (Muranski et al., 2011). Using gene set enrichment analysis, it was showed that the long-live *in vivo* CD4 Th1 cell population derived from CD4 Th17 cells possess a molecular signature of memory cells, including the enrichment of the gene Tcf7 which encodes for TCF-1, suggesting that it could play a role in the long-term maintenance of these cells.

In summary, different factors are involved in the homeostasis of Tm cells. CD8 and CD4 Tm cell survival and proliferation is independent of the interaction of the TCR with self-peptide-MHC. CD8 Tm cell self-renewal homeostatic proliferation in a full compartment is more dependent on IL-15 while their survival is more dependent on IL-7. Ag-experienced CD4 Tm cells required both IL-7 and IL-15 to self-renew in a full compartment, and they present a greater requirement for IL-7 to survive. The TNF family ligand is also involved in the survival of CD4 and CD8 Tm cells. And recently, the Wnt pathway through its nuclear effector TCF-1 has been shown to be involved in CD8 Tm cell self-renewal. The different signals together, could be interacting to modulate the response of the Tm cells to different microenvironments.

Hypothesis

Remarkably, CD8 Tm cells share several characteristics with HSC. First, both cell types use the BM as their reservoir (niche) for their long-term maintenance (Becker et al., 2005; Isern and Mendez-Ferrer, 2011).

Second, Tm cells and HSC partially share a transcriptional profile including some genes known to control self-renewal. Long-term HSCs and memory T and B cells need to be maintained for the lifetime of the organism. It was shown that these cells share a transcriptional program by comparing the gene-expression profiles of naive, effector and memory CD8 T cells with those of long-term, short-term HSCs, and lineage-committed progenitors. The up and down regulation of the transcripts is more similar in HSCs and memory cells compared to the other cells. For example, transcripts which start with higher levels in HSCs and end with lower levels in the more differentiated cells are also higher in memory cells and in the opposite direction from differentiation, go to lower levels in naïve cells. Their common signature suggests that HSCs and memory T and B cells share a self-renewal phenotype (Luckey et al., 2006).

Third, the Wnt pathway is involved in the self-renewal of both cell types and in the case of HSC, Wnt enhances HSC self-renewal likely through an increased expression of *Hoxb4*. It has been shown that the Wnt pathway is involved in the maintenance of both cell types. On one hand, Wnt favors CD8 Tm cell formation and the maintenance of the stem-like characteristics of the population. (Gattinoni et al., 2009; Jeannet et al., 2010; Zhao et al., 2010). While IL-15 signaling is needed for CD8 Tm cell self-renewal (Goldrath et al., 2002); the Wnt pathway can activate Tcf-1 which is linked to the positive regulation of IL-15R and Eomes, thus regulating the proliferative self-renewal capacities of CD8 Tm cells (Zhou et al., 2010). Moreover, deficiency in Tcf-1 results in impaired CD8 Tm cell maintenance and proliferative response after rechallenge (Jeannet et al., 2010; Zhou et al., 2010). Therefore, Wnt could be regulating different pathways that are interrelated for the control of CD8 Tm cell maintenance and proliferative capacities. On the other hand, activation of Wnt signaling expands the pool of long-term HSCs, while blocking Wnt signaling inhibits HSC growth and reduces hematopoietic reconstitution of mice. Moreover, activation of the Wnt signaling in HSCs induces higher expression of *Hoxb4*

(Reya et al., 2003), which is a potent enhancer of HSCs self-renewal expansion (Sauvageau et al., 1995; Thorsteinsdottir et al., 1999). Besides, it has been shown that the *Hoxb* cluster genes are barely (*Hoxb1*) or not express in quiescent human T cells but over induced with activation. Furthermore, when *Hoxb2* or *4* are blocked, the proliferation and activation of human quiescent T cells is drastically inhibited (Care et al., 1994).

In summary, there is now evidence that **the stem cell-like attributes and signaling pathways are present in CD8 Tm cells**, including the signals that regulate their maintenance, proliferation and differentiation (Gattinoni et al., 2012).

Based on these similarities between HSCs and CD8 Tm cells we **hypothesize that genes involved in HSC self-renewal, like *Hoxb4*, will promote CD8 Tm cell expansion**.

Objectives

General objective:

– Evaluate the competitive advantage provided by the overexpression of *Hoxb4* in CD8 Tm cells.

Specific objectives:

– Determine the size of the CD8 Tm cell populations in a transgenic mouse model where *Hoxb4* is overexpressed in lymphoid cells. Evaluate if there is a difference between young and old mice.

– Monitor the engraftment and long-term maintenance of CD8 Tm cells overexpressing *Hoxb4* following their transfer into immunocompetent mice.

– Compare the engraftment and long-term maintenance of CD8 Tm cells overexpressing *Hoxb4* following their transfer into immunodeficient mice.

Material and methods

I.- Mice

All the mice were bred and maintained under specific pathogen-free conditions at the animal facility of the Maisonneuve-Rosemont Hospital Research Center. All protocols were approved by the local animal protection committee.

We used a mouse model were the *Hoxb4* transgene is expressed in lymphoid cells. These mice were generated using the pLIT3 vector as previously described (Bijl et al., 2008). Briefly, downstream of a TCR Vβ promoter, *Hoxb4* murine cDNA was cloned, with immunoglobulin enhancer elements and sequences from the proximal promoter of the lck gene. To provide introns for enhanced expression, the human growth hormone gene containing a frameshift mutation in the coding region was inserted.

Wild type mice C57BL/6 (CD45.2) and B6.SJL (CD45.1) were purchased from Jackson Laboratories (Bar Harbor, ME). *Hoxb4* transgenic mice (CD45.2) were bred to B6.SJL mice to generate combined CD45.1/.2 *Hoxb4* transgenic for transplantation assays.

OT-1 (CD45.1/2) Rag1$^{-/-}$ mice (Hogquist et al., 1994), from Jackson Laboratories (Bar Harbor, ME), were donated from the laboratory of Nathalie Labrecque, and bred to *Hoxb4* (CD45.2) to obtain the compound transgenic mice.

II.- Mice genotyping

To identify the *Hoxb4* transgenic mice, we used genomic DNA from the tail and performed the polymerase chain reaction for the human growth hormone gene (sense sequence: CGGACTGGGCAGATCTTCAA, anti-sense sequence: CCTTGTCCATGTCCTTCCTGAA).

The OT-1 transgenic mice were identified by FACS using the Mix number 1, observing the expression of Vα2 TCR.

III.- *In vitro* generation of CD8 Tm cells

To generate a large numbers of OT1 CD8 Tm cells, spleen cells were stimulated with the ovalbumin peptide SIINFEKL (OVA, from Midwest Biotech). For example: 20 million cells were suspended in 8 ml of medium and stimulated with 0.1 µl/ml of the OVA peptide (10 mg/ml). The stimulation was stopped after 16h by washing the cells. Medium was a preparation of RPMI 1 X (Wisent Inc., St-Bruno, QC, Canada) medium supplemented with 10 % Fetal Bovine Serum, 2 mM L-Glutamine (both Gibco), 100 U/ml penicillin, 100 ug/ml streptomycin (both Wisent Inc.), 10^{-5} M β-mercaptoethanol (Mallinckrodt Baker Inc., Phillipsburg, NJ), sodium pyruvate 1 mM, Hepes 10 mM and non-essential aminoacids 1 X (all from Life Technologies). The medium was changed on day 2. On day 3, the viable cells were separated using a Ficoll gradient (Ficoll Paque^{TM} plus), resuspended at 1 million cells per ml of media and then they were stimulated with 20 ng/ml human IL-15 (Invitrogen) every 2 days (days 3, 5 and 7). To monitor the cellular activation and memory T cell differentiation, the cellular phenotype was followed on days 0, 1, 2, 3, 5, 7 and 9 by staining the cells with antibodies directed against different cell surface markers with the combinations shown in Table 2 and 3, and characterized by Fluorescence Activated Cell Sorting (FACS) analysis. On day 9, cells were prepared for the transplantation assays by washing and resuspending at 3 or 6 million cells per 200 µl of PBS 1 X (no serum).

IV.- CFSE staining

10^7 cells in 1 ml PBS1 X were incubated with 0.1 µl of the fluorescent cell staining dye carboxyfluorescein succinimidyl ester (CFSE, from Lifetechnologies) 5 mM for 10 min at 37°C

protected from light and frequently mixed. Excess CFSE was then removed by adding 1 volume of fetal calf serum or fetal bovine serum. To eliminate the serum and excess CFSE, the recovered cells were washed and resuspended at 3 or 6 million cells per 200 µl of PBS 1 X (no serum) prior to transplantations assay and a sample was kept for FACS analysis.

V.- Antibody staining and FACS analysis

Tm cell surface markers were characterized by FACS performed on LSRII with a UV laser (BD Bioscience). For staining, 1 million cells were resuspended in 50 µl FACSWASH (DMEM without phenol red + 3 % of horse serum + Hepes 30 mM + 0.1 % sodium azide) with the corresponding antibodies at the optimal concentration (determined by titration) and incubated on ice for 30 minutes, protected from light. Cells were then washed and resuspended in FACSWASH before FACS analysis observations on the LSRII. To further analyze the different cell populations, the FlowJo software (Tree Star Inc., Ashland, OR, USA) was used.

Table 2.- Antibodies mixed for the different assays.

MIX	Used for:
1	Transgenic OT-1 mice verification
2	CD8 Tm *in vitro* generation
3	CD8 Tm *in vitro* generation
4	Transplantation to WT normal mice
5	Transplantation in competition into lymphopenic induced mice

Table 3.- List of antibodies used for surface staining.

Antibody	Fluorocrome	MIX	Company	Clone
CD45.1 (Ly5.1)	Pacific blue	4, 5	BioLegend, San Diego, CA	A20
CD45.2 (Ly5.2)	FITC	1, 4	eBioscience, San Diego, CA	104
CD45.2 (Ly5.2)	APC	5	BioLegend, San Diego, CA	104
Vα2 TCR	PE	1	BD Biosciences, Mississauga, ON	B20.1
CD8	APC	1, 3	BioLegend, San Diego, CA	53-6.7
CD8	FITC	2	BioLegend, San Diego, CA	53-6.7
CD8	APCCy7	4, 5	BD Biosciences, Mississauga, ON	53-6.7
CD4	PerCP	1	BioLegend, San Diego, CA	GK1.5
CD19	PECy7	1	BD Biosciences, Mississauga, ON	1D3
CD69	FITC	3	BioLegend, San Diego, CA	H1.2F3
CD44	PECy7	2, 3, 4, 5	BioLegend, San Diego, CA	IM7
CD62L	PE	2, 4, 5	BioLegend, San Diego, CA	MEL-14
Ly6C	Biotin	2, 5	BD Biosciences, Mississauga, ON	AL-21
Ly6C	APC	4	BioLegend, San Diego, CA	HK1.4
1B11 (CD43)	APC	2	BioLegend, San Diego, CA	1B11
Sca-1 (Ly6A/E)	Pacific blue	2	BioLegend, San Diego, CA	E13-161.7
CD127	PerCPCy5.5	2	BioLegend, San Diego, CA	A7R34
Streptavidin	APCCy7	2	BioLegend, San Diego, CA	
Streptavidin	PerCPCy5.5	5	BD Biosciences, Mississauga, ON	

VI.- Transplantation assays to normal WT mice

3×10^6 (in 200 µl of PBS 1 X) of OT-1 or *Hoxb4*/OT-1 transgenic (CD45.1/2) *in vitro* generated CD8 Tm cells were transplanted into WT (CD45.2) sex matched congenic mouse by tail vein injection. LN surgery was performed to follow the same mice after 1, 4 or 8 weeks. The LN, SPL and BM where taken at 3 months when mice were euthanized to characterize the cells by FACS.

VII.- Lymph node surgery

To follow the engraftment of the transplanted cells in the same mice, the LN surgery was performed as previously described (Mathieu and Labrecque, 2012). The brachial or inguinal LN was removed by making a small incision in the skin of an anesthetised (isoflurane 2 %, 1 L oxygen) mouse. The LN were kept on ice and processed to obtain the cells by pressing them against frosted slides. Then, cells were stained and analysed by FACS. It is a superficial surgery and it is rapidly performed allowing the mice to easily recover.

VIII.- Competitive transplantation assays

In vitro generated CD8 Tm cells were stained with CFSE, equally mixed and used in a total dose of $6x10^6$ (in 200 µl of PBS 1 X) per transplanted WT (CD45.1) sex matched congenic mice. Lymphopenic recipient mice for transplantation assays were generated by irradiating WT mice with 600 cGy the day before the transplantation. Transgenic mice were carefully selected to be able to differentiate them by the combination of CD45.1/.2 or expressing only CD45.2. The lymphoid organs were taken from the euthanized mice after 1 day, 1 week, 1 or 3 months and the recovered cells were characterized by FACS.

IX.- Statistical analysis

A two-tailed Student's t-test evaluation was used to obtain the significant difference between cell fractions of mutant mice versus wild type mice. P-value was considered significant when <0.05 indicating difference in cell populations.

Results

I. *Hoxb4* overexpression does not affect the composition of the T cell pools in young and old mice

The pool of T cells can vary with age. With time, the host will have undergone more Ag encounters resulting in accumulation of Tm cells. On the other hand, thymus involution will lead to a decrease in the generation of Tn cells. As a consequence, an older host will have higher numbers of Tm cells and lower numbers of Tn cells than a younger host (Vescovini et al., 2014). In the *Hoxb4* transgenic mice, the transgene is specifically overexpressed in the lymphoid cells, which allows us to study its effect on T cells. We first wanted to evaluate if *Hoxb4* has an impact on the distribution of the different T cell populations, in particular of the naïve and memory populations with age. Thus, we characterized the T cell subpopulations in young and old *Hoxb4* transgenic mice.

Previously we have demonstrated that the *Hoxb4* transgene was expressed in the thymus and spleen (Bijl et al., 2008). We further evaluated whether *Hoxb4* was overexpressed in the naïve T cells and the memory-like T cell phenotype (MP T) (Frison et al., 2013). The expression of the transgene was several folds higher than the endogenous *Hoxb4* expression. The overexpression of *Hoxb4* transgene mRNA compared to WT was 40x in naïve, 7x in MP CD4 T cells, 43x in naïve and 90x in MP CD8 T cells. The highest overexpression was found in CD8 Tm cells (Frison et al., 2013).

FACS analysis of the different T cell populations did not show any significant differences in the proportion of CD4 naïve and CD8 naïve (CD44lo, CD62Lhi), CD4 MP and CD8 MP (CD44hi) T cells within the LN of young (2-3 months) WT and *Hoxb4* mice (**Figure 3**). Similar results were obtained in the SPL and BM (**Figure 4**). As expected, a higher frequency of Tn is observed in LN and SPL from young mice and this hold true for *Hoxb4* transgenic mice (**Figure 4**). On the other hand, CD4 and CD8 MP T cells are in higher percentage in the BM from both WT and *Hoxb4* mice, which is a well-known niche for Tm cells. Furthermore, it is well documented that the Tn cell population decline with age, while Tm cell number is stable. Therefore, the percentage of the different T cell populations in old (>28 months) mice was

compared between WT and *Hoxb4* mice. Both, the WT and *Hoxb4* old mice have a higher percentage of CD4 and CD8 MP T cells and a lower percentage of CD4 and CD8 Tn cells in LN, SPL and BM. Also, the CD4 and CD8 Tn cell percentage is reduced with age in both WT and *Hoxb4* mice while the CD4 and CD8 MP T cell percentage is increased (**Figure 4**). Moreover, we did not observe any differences in the proportions of MP T cells. Altogether, our results suggest that *Hoxb4* overexpression does not affect the intrinsic and extrinsic mechanisms regulating the size of the different T cell pool. We demonstrated that the overexpression of *Hoxb4* is not affecting the steady state T cell homeostasis through the life of the mice.
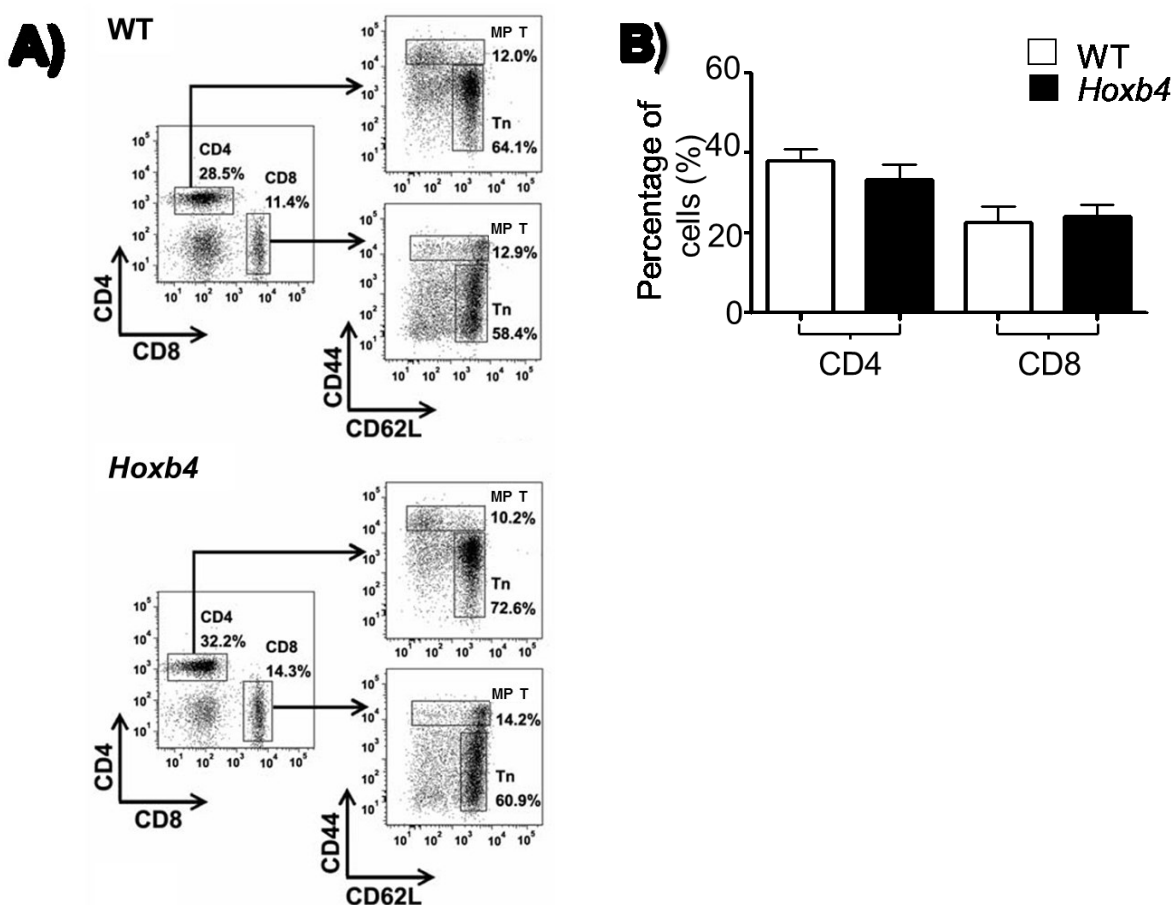
Figure 3.- Similar T cell compartment in WT and *Hoxb4* young adult transgenic mice.
A) Representative FACS profile showing the distribution of naïve (CD44^{lo}, CD62L^{hi}) and memory phenotype (CD44^{hi}) CD4 and CD8 T cell compartments in the lymph node of WT and *Hoxb4* transgenic mice. **B)** *Hoxb4* overexpression does not affect the frequency of CD4 and CD8 T cells within the lymph nodes. The mean +/- standard deviation percentage of CD4 and CD8 T cells is shown. n=6 for WT and 7 for *Hoxb4* transgenic mice. A student test did not reveal any statistical difference between WT and *Hoxb4* transgenic mice. MP T = memory phenotype T cells, Tn= naïve T cells, WT = wild type mice.
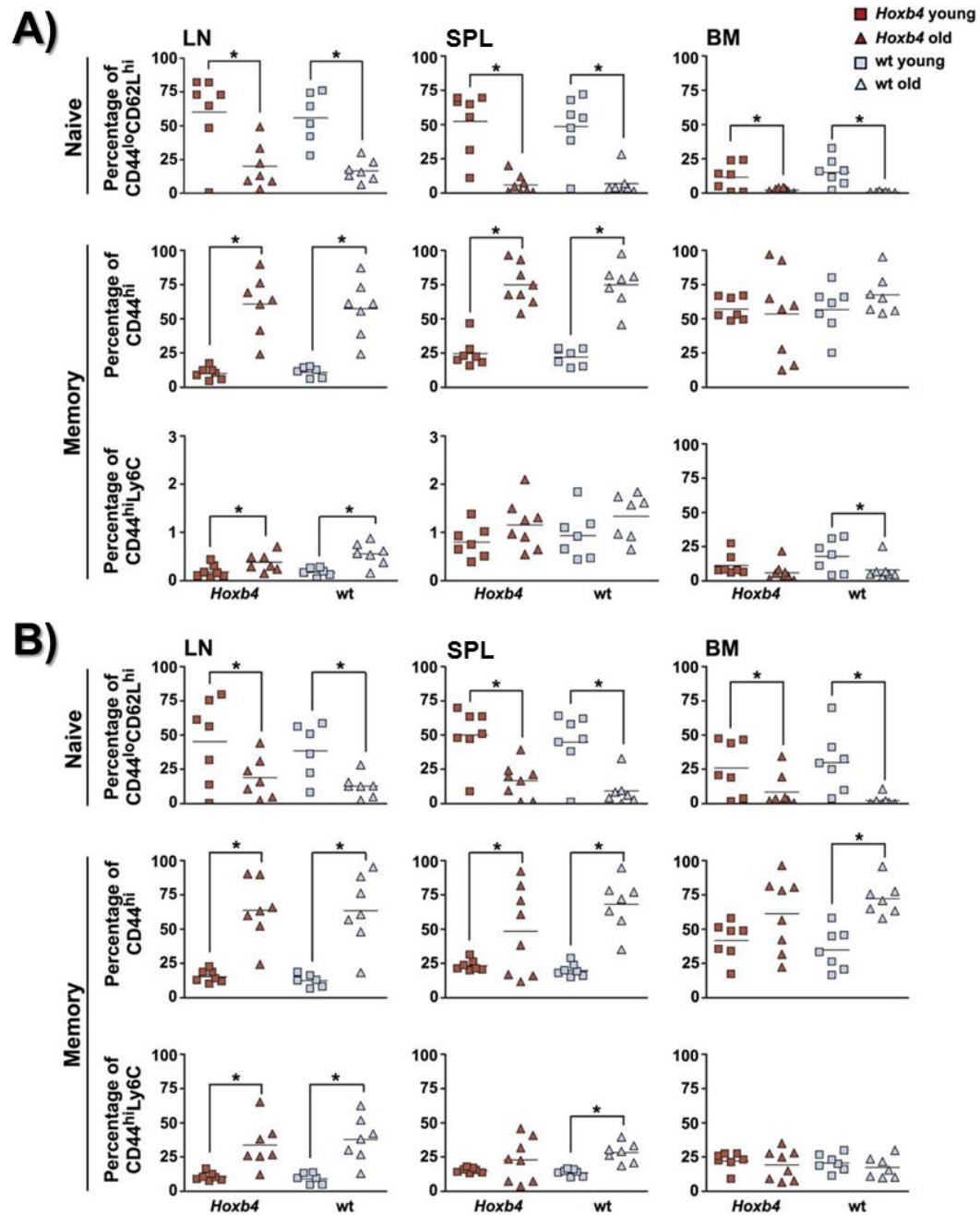
Figure 4.- Similar changes in T cell populations compartment in WT and *Hoxb4* transgenic mice with age.

Scatter plots show the percentage of the naïve and memory **A)** CD4 and **B)** CD8 T cells in the different organs (LN, SPL and BM) from young (3-4 months) and old mice (>28 months). n=6 for WT and 8 for *Hoxb4* transgenic mice. T cell populations are shown as naïve (CD44^{lo}, CD62L^{hi}), MP (CD44^{hi}) and MP T fraction (CD44^{hi}, Ly6C^{hi}). * P<0.05, 2-tailed Student test. BM = bone marrow, LN= lymph node, MP = memory phenotype T cells, SPL = spleen, WT = wild type mice.

II. *Hoxb4* overexpression does not provide an advantage to the engraftment and long-term maintenance of CD8 Tm cells after independent transplantation into immunocompetent mice.

II.I.- *Hoxb4* overexpression does not affect the *in vitro* generation of CD8 Tm cells.

Our results showed that the steady-state T cell homeostasis is not affected by *Hoxb4* overexpression. However, in the transgenic model, it is not possible to evaluate if *Hoxb4* overexpression provides an advantage on the self-renewal of the cells. Thus, we decided to use the transplantation model that could allow us to better evaluate if there is an advantage provide by *Hoxb4* overexpression in CD8 Tm cells. Consequently, we needed to have access to a large number of CD8 Tm cells for transplantations. Therefore, we chose one of the most widely used transgenic TCR model, the OT-1 transgenic mice, to obtain the cells for our *in vitro* CD8 Tm cell generation. In these mice, the CD8 T cells express a TCR specific for the SIINFEKL peptide of ovalbumin presented by the MHC class I molecule H-2K^b. This model allows us to easily produce CD8 Tm cells with a defined specificity and to follow the cell phenotype by FACS all along the *in vitro* generation. Moreover, as the CD8 Tm cells generated with this model were expressing different leukocytic surface antigens, it was easier to follow the transplanted cells *in vivo*.

We first needed to corroborate if the *Hoxb4* transgenic CD8 T cells were able to respond to their antigen and to differentiate into Tm cells. *Hoxb4* transgenic mice were crossed with the OT-1 transgenic mice. The resulting double transgenic mice were used to generate the *in vitro* CD8 Tm cells specific for SIINFEKL-K^b and *Hoxb4* overexpression. We monitored both, WT and *Hoxb4* cultures during the 9 days required for *in vitro* generation of CD8 Tm cells. There

was no difference in the absolute numbers of CD8 Tm cells that were generated, comparing WT and *Hoxb4* overexpressing cells, as shown in **Figure 5A**. We also monitored the expression of different surface markers during the *in vitro* generation. An example of the expression of CD44 and CD62L on day 0, 3 and 9, is shown in **Figure 5B**. We evaluated if the CD8 Tn cells get activated normally when they overexpress *Hoxb4*. We observed at day 2 a similar increased in the expression of CD69 and CD44, and a decreased of CD62L expression in WT and *Hoxb4* CD8 T cells. Then, we evaluated if their differentiation was affected by *Hoxb4* overexpression, by following the cells surface phenotype of the *in vitro* culture by FACS overtime (days 3 to 5). No phenotypic differences were observed between WT and *Hoxb4* T cells. In both cases, we observed a rapid loss of CD69 and CD62L expression, and a gain of CD44 and 1B11 expression. We also observed an increased expression of Sca1, CD127 and Ly6C over time in both WT and *Hoxb4* T cell cultures. Finally, at day 9 (**Figure 6**), we evaluated if *Hoxb4* overexpression was affecting the memory phenotype of *in vitro* CD8 Tm cells. Both cultures presented no expression of CD69, and compared to the initial CD8 T cells, higher expression of CD44, Sca1, 1B11, CD127, Ly6C and regained a higher expression of CD62L as expected for CD8 Tm cells. In summary, there were no differences in the phenotype of WT and *Hoxb4* CD8 Tm cells during *in vitro* generation. These results suggest that *Hoxb4* overexpression does not interfere with the *in vitro* generation of CD8 Tm cells. At day 9 of *in vitro* generation, the resulting CD8 T cells are considered as a central memory (CD44$^+$, CD62L$^+$, Ly6C$^+$) population that will end its differentiation after transplantation, possess the ability to persist *in vivo* and to mount a response to Ag reencounter.

A)

B)
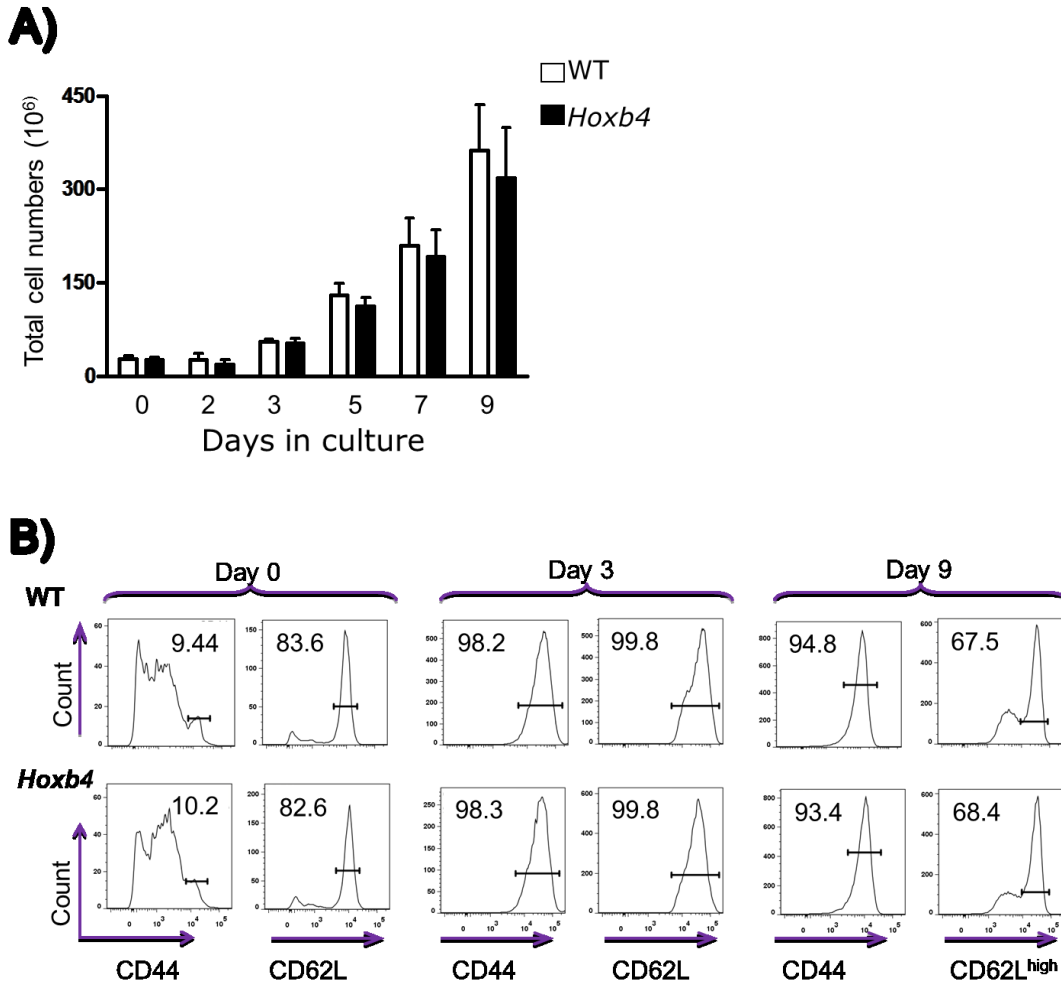
Figure 5.-Similar number and phenotype of WT and *Hoxb4* CD8 T cells during *in vitro* differentiation culture.

Briefly, the splenocytes from OT-1 or *Hoxb4*/OT-1 transgenic mice were stimulated with the OVA peptide SIINFEKEL for 16 h. Cells were washed and keep in culture up to 3 days. At day 3, viable cells were enriched using a Ficoll gradient and culture with human IL-15 up to day 9. Cells surface staining was performed at different time points do follow T cell activation and differentiation throughout the culture. **A)** Number of CD8 T cells recovered during the *in vitro* culture. The data is from 4 independent experiments and a 2-tailed Student test did not reveal any statistical differences between WT and *Hoxb4* cultures. **B)** Expression of CD44 and CD62L by CD8 T cells during the *in vitro* differentiation culture. Representative FACS profile gated on CD8$^+$ T cells are shown. The number on the FACS profile indicate the percentage of cells positive for the cell surface marker. OVA = ovalbumin, WT = wild type mice.
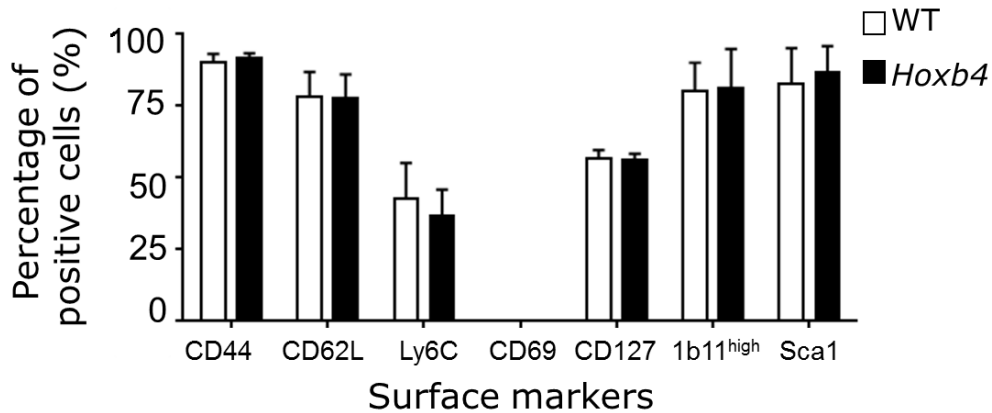
Figure 6.- Similar expression of cell surface markers on WT and *Hoxb4* CD8 Tm cells generated *in vitro*.

Average percentage of surface markers in CD8 Tm cells at day 9 of *in vitro* culture (n=6). No statistical significant differences were observed comparing WT vs. *Hoxb4* overexpressing cells. 2-tailed Student test. WT = wild type mice.

II.II.- *Hoxb4* overexpression does not affect the transplantation of the *in vitro* generated CD8 Tm cells into normal mice

Then, we wanted to know if the *in vitro* generated CD8 Tm cells overexpressing *Hoxb4* were able to enter the lymphoid organs (LN, SPL, BM) and to compete with the WT cells present in a full niche. Normally, after the generation of the CD8 Tm cells in the body, these cells will compete with the population that is already stablished. The *Hoxb4* or WT *in vitro* generated CD8 Tm cells (CD45.1/2) were independently transplanted into WT mice (CD45.2) and followed by LN surgery after 1 week, 1 and 2 months. The LN surgeries allow us to follow the transplanted cells in the same mice through time. The cellular phenotype, comparing WT and *Hoxb4* transplanted cells remained similar during the course of the experiment (**Figure 7A**)

retaining characteristics of central memory CD8 T cells with high levels of CD44, CD62L and Ly6C. With the LN surgeries performed on the normal mice, we observed that WT transplanted cells had a tendency to be present in higher numbers compared with the *Hoxb4* CD8 Tm cells, although no statistical significance was reached (**Figure 7B**). Furthermore, no statistical difference in numbers was found in lymph nodes, spleen and bone marrow, which is considered as their main niche, after 3 months when mice were euthanized (**Figure 7C**). Hence, *Hoxb4* overexpression does not confer an advantage for the entry and maintenance of CD8 Tm cells in a full niche.
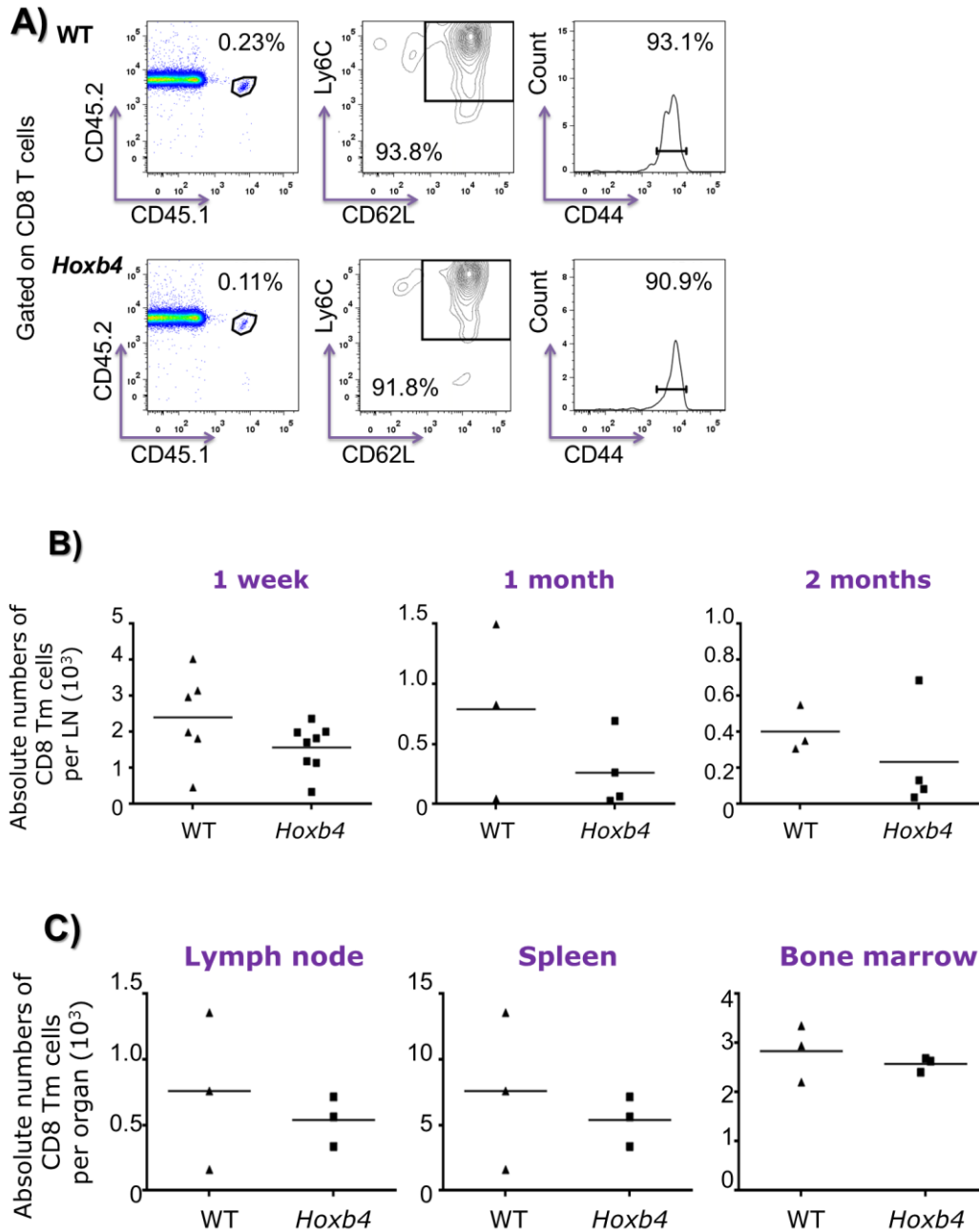
Figure 7.- Comparable CD8 Tm cell population are maintained after independent transplantation into immunocompetent mice.

$3x10^6$ of OT-1 or *Hoxb4*/OT-1 transgenic *in vitro* generated CD8 Tm cells were transplanted into WT sex matched congenic mouse. **A)** Representative FACS profile of WT and *Hoxb4* CD8 Tm cells obtained from LN surgeries after 1 week. The values in the graph correspond to the percentage of the CD8 T cells that are positive and high expressing the corresponding marker.

B) Absolute numbers of WT and *Hoxb4* CD8 Tm cells recovered from LN surgeries after transplantation at 1 week (n =6 for WT and 8 for *Hoxb4* transgenic mice), 1 and 2 months (n=3 for WT and 4 for *Hoxb4* transgenic mice). **C)** Absolute numbers of *Hoxb4* (n=3) and WT (n=3) CD8 Tm cells recovered from different organs (LN, spleen and bone marrow) at 3 months after transplantation into immunocompetent mice. No significant differences were observed. 2-tailed Student test. LN = lymph node, WT = wild type mice.

II.III.- *Hoxb4* and WT CD8 Tm cells home properly to secondary lymphoid organs and tertiary sites.

We observed that the WT transplanted cells tend to be conserved in higher numbers than the *Hoxb4* CD8 Tm cells therefore, we decided to explore if the difference in the early homing capacity of the cells could be one of the reasons. To evaluate the capacity of *Hoxb4* overexpressing cells to early home to different organs, the *in vitro* generated cells were stained with CFSE, independently transplanted into WT normal mice and sacrificed the next day for analysis. We dissected the SPL, BM, peritoneal cavity (PC), skin draining lymph nodes (SDLN) and mesenteric lymph nodes (MLN) to evaluate the presence of the transplanted cells by FACS. We found that the percentage of WT and *Hoxb4* CD8 transplanted Tm cells were not significantly different except in the SDLN where *Hoxb4* CD8 Tm cells were found in higher numbers compared to WT transplanted cells (**Figure 8**). These results showed that cells overexpressing *Hoxb4* were capable to migrate to the different organs. Therefore, the migration capacity of *Hoxb4* overexpressing CD8 Tm cells is not the reason why they tend to be maintained in lower numbers compared to WT transplanted cells.
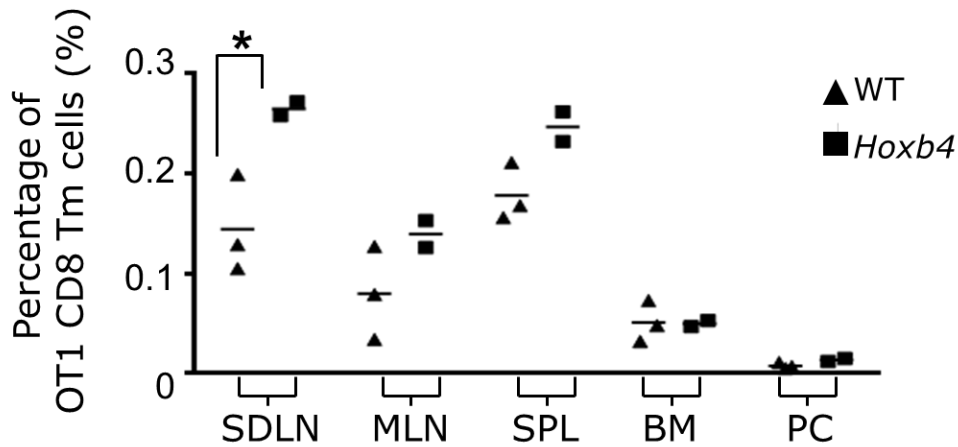
Figure 8.- Similar migratory capacity of *in vitro* generated WT and *Hoxb4* OT-1 CD8 Tm cells following their adoptive transfer into immunocompetent mice.

3×10^6 *in vitro* generated WT (OT-1) or *Hoxb4*/OT-1 CD8 Tm cells were labeled with CFSE and transplanted into WT sex-matched congenic mice. *Hoxb4* (n=2) and WT (n=3) CD8 Tm cells (CFSE$^+$, CD8$^+$) were recovered from SDLN, MLN, SPL, BM and PC after 1 day of transplantation into immunocompetent mice. The percentage of CD8 Tm cells recovered from the different organs is similar except for SDLN. *p<0.05, 2-tailed Student test. BM = bone marrow, MLN = mesenteric lymph node, PC = peritoneal cavity, SDLN = skin draining lymph node, SPL = spleen, WT = wild type mice.

III.   *Hoxb4* overexpression does not provide an advantage to the engraftment and long-term maintenance of CD8 Tm cells after competitive transplantation into lymphopenic mice.

The independent transplantation of CD8 Tm cells into immunocompetent mice did not reveal any advantage provided by *Hoxb4* overexpression. In immunocompetent mice the available niche is limited, because the mice have normal number of the different cells types competing to survive in their niche. Therefore, the cells can only slowly self-renew. We decided

to evaluate if *Hoxb4* could provide an advantage on the self-renewal of the cells when transplanted into an empty niche, where they do not have to compete with other cells already established in the niche. In order to evaluate if the *Hoxb4* CD8 Tm cells could have an advantage over WT CD8 Tm cells under lymphopenic conditions, the WT and *Hoxb4 in vitro* generated CD8 Tm cells were labeled with CFSE and transplanted in competition in a ratio of 1 to 1 into lymphopenic mice. A 600 cGy non-lethal irradiation of WT mice the day before the transplantation allow us to generate a temporary empty niche, these lymphopenic mice are able to recover and to start the generation of their blood cells from HSCs. To monitor the contribution of the transplanted cells, peripheral blood was taken and organs (LN, SPL and BM) were processed after euthanasia at 1 day, 1 week, 1 and 3 months, and analyzed by FACS for CD45.1 and CD45.2 expression to differentiate the cells. Upon mice genotyping, depending on which mice was overexpressing *Hoxb4*, CD45.1 or CD45.2 was selected to follow the WT or the *Hoxb4* cells during transplantation.

Our results (**Figure 9A**) showed that the absolute numbers of CD8 Tm cells recovered from lymph node, spleen and bone marrow are not significantly different between the *Hoxb4* and WT transplanted cells at any time point. However, there was a trend for a lower recovery of *Hoxb4* CD8 Tm cells when compared to WT. We also confirmed that there were no differences in the cell numbers present in the skin draining and mesenteric lymph nodes after 3 months. However, when the WT and *Hoxb4 in vitro* generated CD8 Tm cells are mixed prior to transplantation, the mix of *Hoxb4*/WT cells is not a perfect 50:50 ratio. Therefore, we corrected the percentage of the recovered cells according to the real transplantation percentages (nearby 55 to 45%) measured by FACS in the cellular mix before transplantation. With this correction (**Figure 9B**) we evaluated the ratio of contribution of *Hoxb4*/WT cells to the different organs. At day 1 post transfer *Hoxb4* CD8 Tm cells seems to stay more in the blood and could have a delay to enter to the other lymphoid organs compared to the WT transplanted cells. However, at 1 week, they are almost at the same levels in all the organs. After 1 and 3 months, the percentage of *Hoxb4* CD8 Tm cells that are found in the LN and SPL gets lower compared to the WT transplanted cells. Interestingly, in the BM, we found that the levels of *Hoxb4* CD8 Tm cells were similar to those of WT transplanted cell. Therefore, *Hoxb4* does not enhance CD8 Tm cell numbers in the different organs, but seams to lower in LN and SPL.

We also evaluated if the CD8 Tcm markers continued to be expressed with time. After 1 month, we found that the expression of the CD44, CD62L and Ly6C markers were similar between the WT and the *Hoxb4* CD8 Tm transplanted cells (**Figure 10A**). We observed similar results at each time point of observation and until the end of the experiment at 3 months as shown in **Figure 10B**. Therefore, the phenotype of the *Hoxb4* CD8 Tm cells remained similar to the WT transplanted cells.

The empty niche allows us to provide in abundance all the signals required for the cells to self-renew. Besides measuring the number of cells recovered, to evaluate if *Hoxb4* could provide a self-renewal advantage compared to the WT transplanted cells under this condition, we decided to stain the cells with CFSE. The progressive dilution of CFSE was used to observe the cellular division. Our results are display in the LN FACS profile **Figure 11A**. Marked peak of division were not always observed in the experiments. Therefore, we did not quantify the division peak, but clearly all the cells have divided. We overlapped the gate profiles from day one on the other days and observed the progressive dilution of the CFSE at day 8. As cellular division continues, we were not able to detect the CFSE in the majority of the cells at day 30. The mean fluorescence intensity was measured on the gates (bold marker in **Figure 11A**) with the majority of the cells from each time point. We use this measure to plot in **Figure 11B** the progressive dilution of the CFSE in the CD8 Tm cells at 1, 8 and 30 days, from the different organs (LN, SPL and BM). We observed that the progressive dilution of the CFSE was similar between *Hoxb4* and WT CD8 Tm transplanted cells. Hence, the division of both cell types was similar. For all the reasons mentioned before (contribution, phenotype and division), we conclude that *Hoxb4* does not enhance CD8 Tm self-renewal.
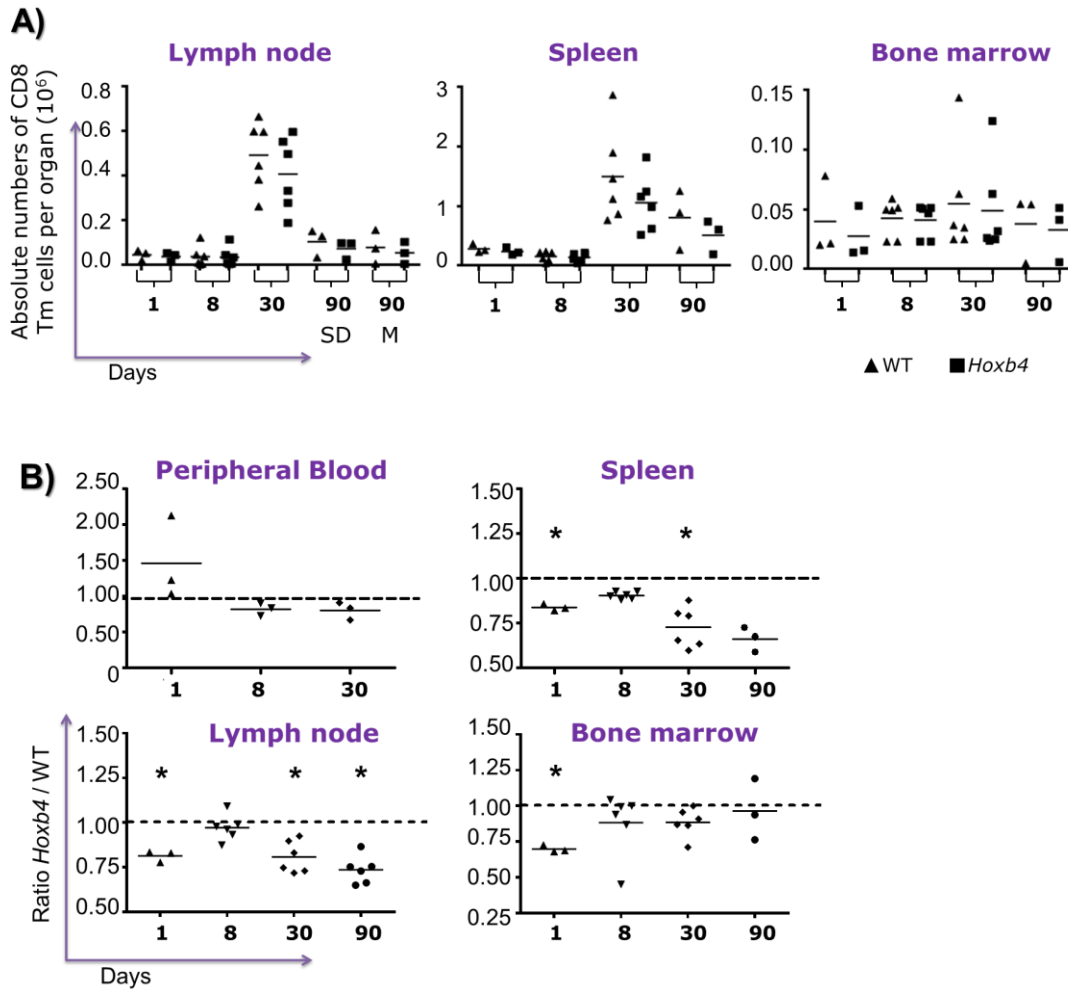
Figure 9.- Contribution analysis of *Hoxb4* and WT CD8 Tm cells after competitive transplantation into lymphopenic mice reveled no advantage conferred by *Hoxb4*.

A total dose of 6×10^6 *in vitro* generated CD8 Tm cells (1:1), stained with CFSE, were transplanted into lymphopenic (600 cGy irradiated) mice. **A)** Absolute numbers of *Hoxb4* and WT CD8 Tm cells recovered from different organs at 1, 8, 30 and 90 days post transfer into lymphopenic mice. No significant differences were observed. 2-tailed Student test. **B)**. CD8 Tm cell contribution to the different organs. *Hoxb4* and WT CD8 Tm cells were recovered after 1, 8, 30 and 90 days from peripheral blood, LN, SPL and BM. First, a correction for the initial percentage evaluated by FACS prior to transplantation was done. Then, a ratio *Hoxb4*/WT. The line at value 1 represents equal contribution. *$p<0.01$, 2-tailed Student test. BM = bone marrow, LN = lymph node. M= Mesenteric LN, SD= skin draining LN, SPL = spleen, WT = wild type mice.
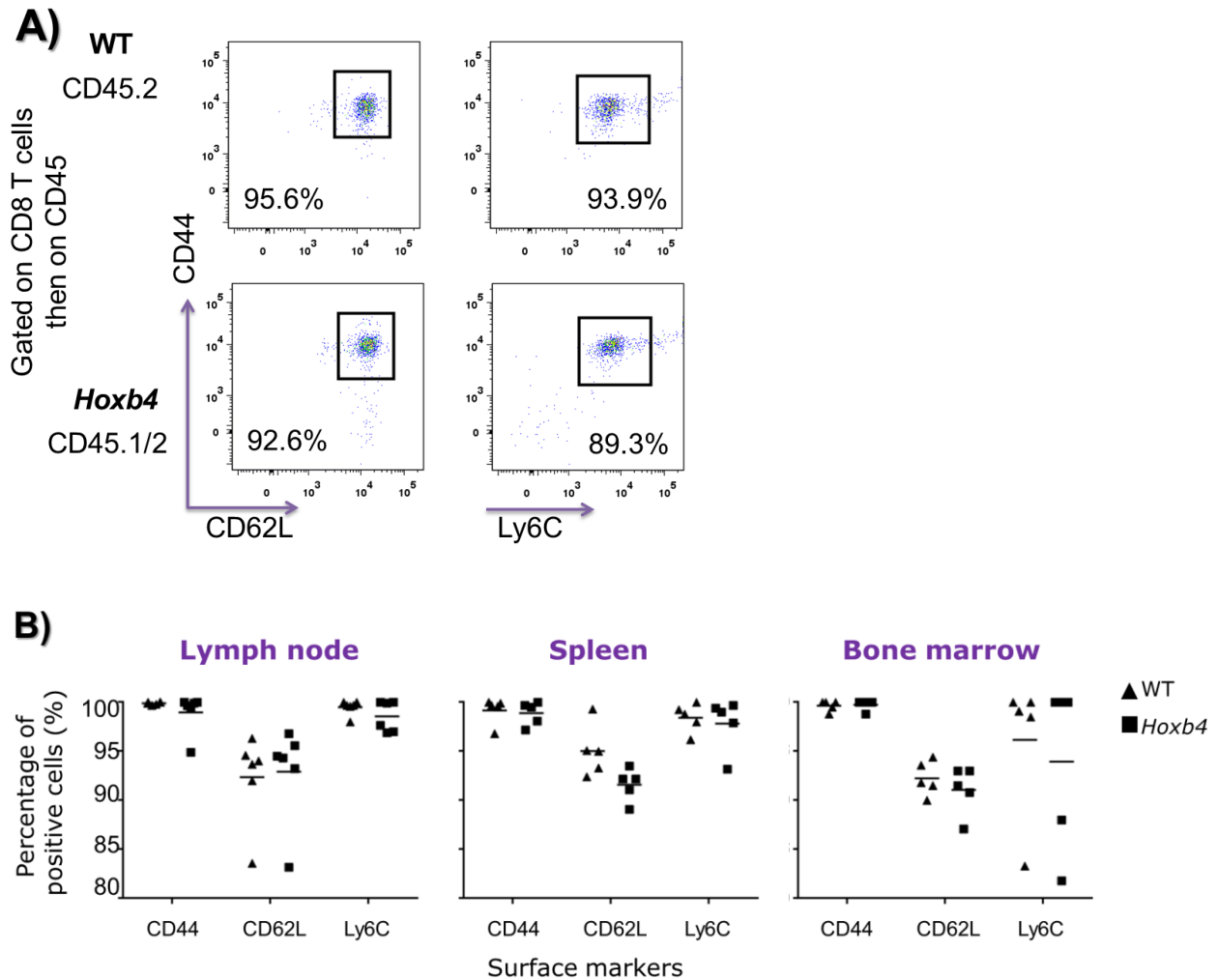
Figure 10.- Similar phenotype of *in vitro* generated WT and *Hoxb4* CD8 Tm cells after competitive transplantation into lymphopenic mice.

A total dose of 6×10^6 *in vitro* generated CD8 Tm cells (1:1), stained with CFSE, were transplanted into lymphopenic (600 cGy irradiated) mice. CD45.1 and CD45.2 were used to differentiate between WT and *Hoxb4* CD8 Tm cells. **A)** Representative LN FACS profile of *Hoxb4* and WT CD8 Tm cells recovered after 1 month of transplantation in competition. The values in the graph correspond to the percentage of the CD8 Tm cells that are double positive for the corresponding marker. **B)** Percentage of CD8 Tm recovered cells expressing surface markers (CD44, CD62L and Ly6C) after 3 months from LN, spleen and bone marrow. No significant differences were observed. 2-tailed Student test. WT = wild type mice, LN = lymph node.
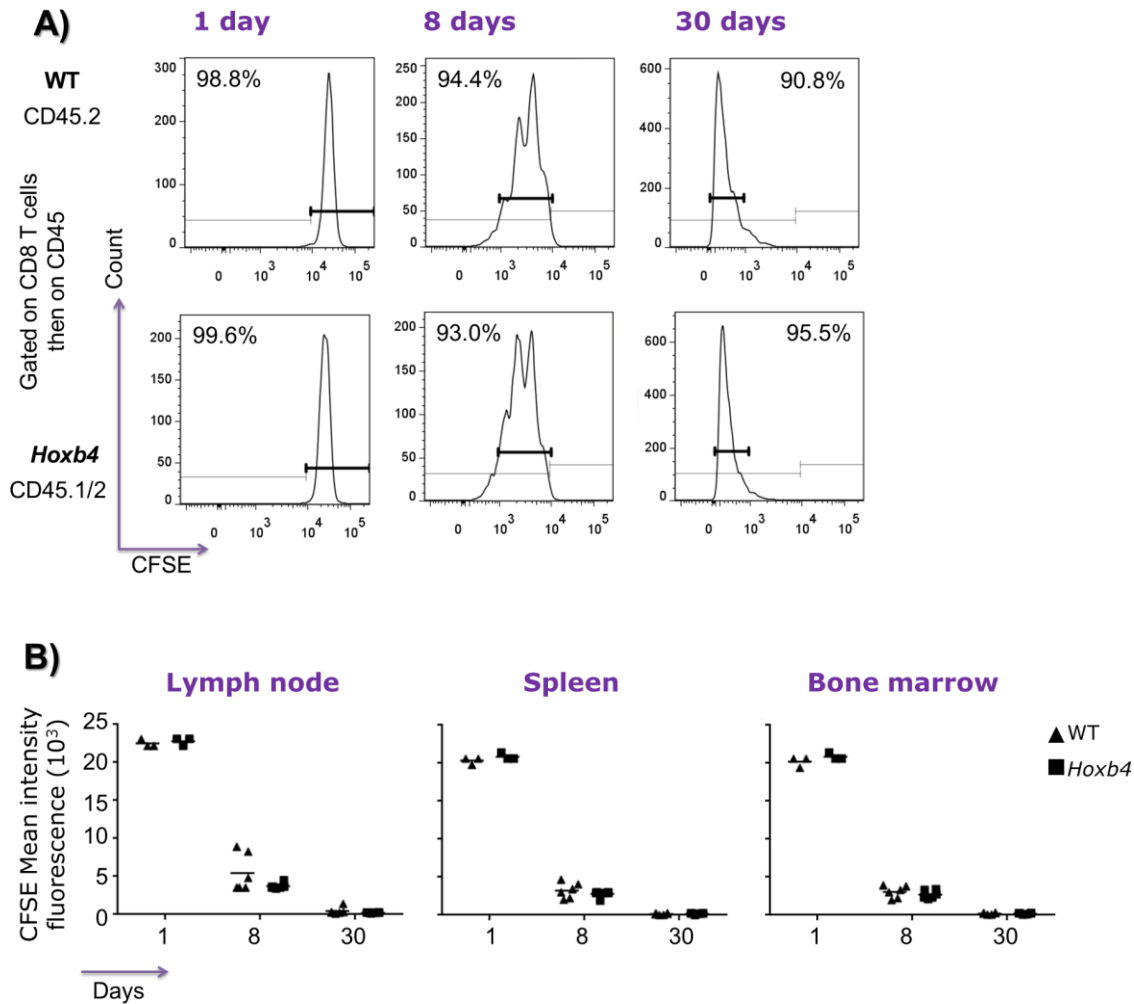
Figure 11.- Similar CFSE dilution after competitive transplantation of WT and *Hoxb4 in vitro* generated CD8 Tm cells into lymphopenic mice.

A total amount of $6x10^6$ *in vitro* generated CD8 Tm cells (1:1), labelled with CFSE, were transplanted into lymphopenic (600 cGy irradiated) mice. After gating on CD8 T cells, CD45.1 and CD45.2 were used to differentiate between WT and *Hoxb4* CD8 Tm cells. **A)** Representative FACS profile of the CFSE staining in the *Hoxb4* and WT CD8 Tm cells recovered from the LN. The bold marker showed the majority of the cells on each graph. These markers were used to measure the mean fluorescence intensity which is indicated on the plot. The marker from day 1 was copied to the other days as a light line to observe the progressive dilution of the CFSE. **B)** Compilation of CFSE mean fluorescence intensity within in *Hoxb4* and WT CD8 Tm cells from different organs at different times post-transplantation. No significant differences were observed. 2-tailed Student test. WT = wild type mice, LN = lymph node.

Discussion

Our results showed that *Hoxb4* overexpression in T cells does not affect the homeostasis of CD8 T cells as we did not observe any difference in the proportion of the naïve and memory T cell populations. We also showed that adoptive transfer of *Hoxb4* overexpressing CD8 Tm cells did not enhanced the long-term contribution of CD8 Tm cells to the memory pool.

Our laboratory has previously shown that *Hoxb4* is overexpressed in naïve and memory T cells from *Hoxb4* transgenic mice and markedly increased in MP CD8 T cells (Frison et al., 2013). In normal mice, with age the CD8 Tm cell pool enlarge, while CD8 Tn cell number decrease (Vescovini et al., 2014). Besides, mice kept under pathogen free conditions develop a memory T cell-like population (Vos et al., 1992). In mice with *Hoxb4* deficiency, HSC generation and lineage distribution is not affected (Brun et al., 2004). In the case of our mouse model overexpressing *Hoxb4*, they did not develop any leukemia, so we considered that *Hoxb4* is not affecting the normal regulation of the cells. As expected, the transgenic mice maintain normal distribution of the T cell populations. With age, as in normal mice, a reduction on the Tn cell compartment and an increase on the MP T cell population was observed in the *Hoxb4* transgenic mice. These results can be explained comparing results found in HSCs. The overexpression of *Hoxb4* does not override the mechanisms controlling the normal pool size limit of HSCs (Thorsteinsdottir et al., 1999). We can say that the T cells overexpressing *Hoxb4* are behaving like the normal cells in any normal mice. Most likely, the mechanisms that control the population numbers remain intact in this model. We conclude that the overexpression of *Hoxb4* does not negatively affect T cell homeostasis during the life of the mice. However, studying mice overexpressing *Hoxb4* in all T cells does not answer if the CD8 Tm cells could have a proliferative advantage compared to WT cells.

To address this question, we first generate *in vitro* the CD8 Tm cells from the OT-1 and *Hoxb4* double transgenic mice. The *Hoxb* cluster is almost not expressed in human quiescent T cells, while induced with activation (Care et al., 1994). As we were overexpressing *Hoxb4*, which is normally occurring during T cell activation, there could be an enhanced activation response. We did not expect that because in the *Hoxb4* overexpressing mice, the T cell populations remained normal, suggesting that the activation mechanism should not be affected. As expected, the overexpression of *Hoxb4* did not interfere with the *in vitro* generation of CD8

Tm cells. These results show that the cells keep their ability to give a normal response following antigen encounter.

To evaluate if *Hoxb4* could provide a competitive advantage to the CD8 Tm cells, we proceeded to monitor the engraftment and long term maintenance of the *in vitro* generated cells in transplantation assays. The Wnt pathway induces the expression of *Hoxb4* in HSCs (Reya et al., 2003), and *Hoxb4* is a powerful enhancer of HSCs self-renewal (Sauvageau et al., 1995; Thorsteinsdottir et al., 1999). On the other hand, the Wnt pathway promotes CD8 Tm cell formation and maintenance (Zhao et al., 2010); and the CD8 Tm cells generated by Wnt signaling possess a higher ability to self-renew (Gattinoni et al., 2009). Moreover, Wnt-Tcf-1 pathway is necessary for the maintenance of CD8 Tm cells (Zhou et al., 2010). In summary, Wnt can control the CD8 Tm cell self-renewal and it has been associated to a higher self-renewal of HSCs trough *Hoxb4*. Thus, we were expecting that *Hoxb4* overexpression in CD8 Tm cells would enhance their self-renewal, but this was not observed. It could be that in CD8 Tm cells, unlike HSCs, the Wnt signaling does not go through *Hoxb4*. However, we cannot exclude that *Hoxb4* could be modulating the self-renewal abilities of CD8 Tm cells depending on other cellular context for example, after re-challenge or when the cells homeostatic proliferate once the re-challenge has passed; because it was shown that Wnt-Tcf-1 pathway also depend on the cellular situation like activation, differentiation and upon re-challenge (Paley and Wherry, 2010; Zhou et al., 2010). Moreover, Wnt signaling has also been associated with outcomes that differ with its self-renewal effect on HSC, depending on the expression levels. Elevated expression is associated with ageing and exhaustion (Florian et al., 2013; Luis et al., 2011). Therefore, it could be that the *Hoxb4* overexpression in CD8 Tm cells, instead of improving self-renewal of the cells, it is inducing a state similar to ageing or exhaustion. However, CD8 Tm cells overexpressing *Hoxb4* are maintained in the BM of the mice 3 months after transplantation without difference compared to WT transplanted cells. Also they present a similar progressive dilution of the CFSE staining, leading us to believe that the cells are not exhausted. With our results, we can conclude that *Hoxb4* overexpression is not interfering with the CD8 Tm cell in two cellular contexts: their generation and their long-life maintenance in normal conditions; but we do not know what would happen after the cells re-encounter the Ag. It could be possible that after re-challenge, the overexpression of *Hoxb4* could conferee an advantage in the cellular

maintenance. To find this answer, the *Listeria monocytogenes* model could be used to re-challenge the mice transplanted with WT and *Hoxb4* overexpressing CD8 Tm cells. This is a well-known controlled model, we can use a non-lethal dose to follow the response and observe the behavior of our cells for long time after the infection. This experiment could also give us information to verify senescence, as senescent cells would not be able to optimally respond to a re-challenge.

Different factors (including signaling pathways and cytokines) control CD8 Tm cell homeostasis, balancing and precisely regulating memory cell proliferation and death rate, in order to keep a stable number of cells under normal conditions (Boyman et al., 2009; Nolz et al., 2012). Transplantation assays in immunocompetent mice demonstrated no advantage of *Hoxb4* overexpression on CD8 Tm cell maintenance. In this setting, our CD8 Tm cells overexpressing *Hoxb4* entered and competed with the existing Tm cells. We considered that due to the limited resources that are available in the normal mice, as their niche is full, the transplanted cells are only being maintained. In the case of HSCs, the experiments where an advantage was found were done under lethal irradiation conditions, which conferred hematopoietic proliferative stress to the transplanted cells because the niche is empty. Therefore, in the second model that we used, we could give more resources for the cells to expand by giving empty niches. So we transplanted in competition the *in vitro* generated *Hoxb4* and WT CD8 Tm cells into irradiated mice. However, we did not find the expected proliferative advantage from *Hoxb4* overexpression. Yet, we considered that the cells were proliferating in a similar way, due to the comparable progressive dilution of the CFSE staining. BrdU staining could be used to verify the proliferation capacity of the cells. From HSCs overexpressing *Hoxb4*, and from the observation done in the *Hoxb4* transgenic mice, our results could be interpreted as if the cells are maintaining normal proliferative capacities even with higher available resources. We can conclude that *Hoxb4* is not affecting the self-renewal capacity of the CD8 Tm cells, but they seem disadvantaged.

Our results from the transplantation experiments into immunocompetent or lymphopenic mice showed a tendency of *Hoxb4* overexpressing cells to be maintained in the LN or SPL at lower numbers compared to the WT transplanted CD8 Tm cells. The generation of death-intermediate memory T cells (T_{DIM}), as a result from cellular division, has been linked to the

control of the CD8 Tm cells numerical stability (Nolz et al., 2012); the proliferating CD8 Tm cells generate the T_{DIM} cells that are apoptotic and nonfuntional. Moreover, besides its proliferative effect in HSCs, *Hoxb4* has been suggested to have a different role depending on the cell type, even promotion of apoptotic pathways in some embryonic structures (Morgan et al., 2004). Therefore, another reason why we found lower numbers of *Hoxb4* CD8 Tm cells compared to WT could be that the proliferation of our cells leads to the generation of T_{DIM} cells. To further investigate this, we could stain the cells with Annexin V to verify cellular apoptosis and test functionality of the recovered cells by stimulation with PMA/ionomycin to measure cytokine production.

The lower contribution of *Hoxb4* overexpressing CD8 Tm cells compare to WT into the LN or SPL could be due to cellular death (as a consequence of their differentiation into T_{DIM}) or their migration to other niches. Therefore, it would be interesting to verify if the *Hoxb4* overexpressing CD8 Tm cells are differentiating into other subpopulations and thus migrating to other organs. We transplanted *in vitro* generated CD8 Tm cells with central memory characteristics according to the high expression of CD44, CD62L (which allows them to migrate through LNs) and non-expression of activation marker CD69 at day 9 of *in vitro* generation. However, Tcm cells may possibly differentiate into Tem (CD44 high, CD62L low) or stem like Tm (CD44 low, CD62L high) (Gattinoni et al., 2012; Yu and Anasetti, 2005; Zhang et al., 2005). The extra-lymphoid CD8 Tem cell populations have been described to possess different characteristics compared to their SPL counterparts and preferentially reside in non-lymphoid tissues (Masopust et al., 2001). More interestingly, the resident CD8 Tm cell populations from different non-lymphoid organs are being studied and are associated with CD69 expression and non-recirculation trough lymphoid organs (Hofmann and Pircher, 2011; Masopust et al., 2010; Sathaliyawala et al., 2013; Schenkel et al., 2013). In our results cells retain high expression of CD44, CD62L and Ly6C surface markers. Thus, we did not observe the conversion to effector (CD62L low) or stem like (CD44 low) CD8 Tm cells in the BM, LN or SPL. We did not evaluate the expression of CD69 in the CD8 Tm cells after transplantation. As we did not evaluate any non-lymphoid organ, we cannot completely exclude the possibility that CD8 Tm cells could differentiate, migrate into non-lymphoid organs and stay there; which could explain the lower contribution found in the LN and SPL. Therefore, we should evaluate the contribution

of *Hoxb4* overexpressing CD8 Tm cells to non-lymphoid organs like liver, lungs or intestinal epithelium; and if we found our cells into any of this organs, it would be interesting to further characterize them in order to know if they are differentiating into Trm cells and acquiring features that allow them to rather stay in the organ and not recirculate to lymphoid organs.

For CD8 Tm cells, it has been shown that the BM is their preferred proliferation site, it contained a major pool of the most actively dividing cells and this division is abrogated in IL-15 deficient mice (Becker et al., 2005). Hence, the BM is the most important niche for the CD8 Tm cells. Our results show the preferred localization in BM over LN or SPL for the *Hoxb4* overexpressing CD8 Tm cells. After transplantation in competition into lymphopenic mice, BM is the only organ where we found similar levels of cells during the follow up of the transplanted mice and until 3 months. As the BM provides IL-15 for the Tm cell homeostatic proliferation, this could mean that *Hoxb4* overexpressing CD8 Tm cells are more dependent on cytokine signaling for their proliferation. It would be very interesting to know what happens when a proliferation signal like IL-15 is missing. By transplanting the *in vitro* generated CD8 Tm cells into IL-15 deficient mice, we could find if the *Hoxb4* overexpression is truly dependent on this signal or if it can confer an advantage by expanding the cells overcoming the missing signal.

In our model, the *Hoxb4* overexpressing mice, the CD8 T cells present a higher expression of *Hoxb4* than the CD4 T cells. Studies performed by us to analyze the CD4 MP T cells overexpressing *Hoxb4*, have shown that in short-term competitive repopulation, WT and transgenic cells contributed equally to LN and BM but WT dominated over *Hoxb4* in SPL. In medium-term competitive repopulation, WT cells presented a higher contribution over *Hoxb4*. With further characterization of the cell surface markers, it was found that *Hoxb4* might indeed favor self-renewal of CD4 MP T cells, but only for the CD62L high cell subpopulation (Frison et al., 2013). Our results with CD8 Tm cells show that WT cells seem to be similarly dominating over the transgenic cells in general. We did not found differences in the surface markers studied, but we cannot exclude that the *Hoxb4* overexpressing CD8 Tm cells could be differentiating and migrating to other organs. We have also to consider that while CD4 MP T cell populations were sorted and contained a mix of both effector and central Tm cells, while we generate *in vitro* CD8 Tcm cells. Although the OT1 model to produce CD8 Tm cells is widely accepted and used, we

cannot exclude that the cells generated *in vivo* could present a different behavior. It could also be possible that *Hoxb4* confers a different proliferative capacity to others subpopulations of CD8 Tm cells. Thus, we could generate an *in vivo* response, sort the CD8 Tm cell populations and transplant them into lymphopenic mice to evaluate their self-renewal capacity.

The balance between the quantity, quality and function of the CD8 Tm cells confers the ability to better respond when an antigen is re-encountered. For example, vaccination strategies attempt to produce a good quantity of Tm cells that will support the defense against the recurrent infection. If we can maintain more CD8 Tm cells, they will be able to massively respond after a secondary challenge. For example, when CD8 Tm cells that respond to Ag expressed by recurrent pathogens or even to cancerous cells, a method to maintain a good quantity of the CD8 Tm cells would be an advantage in order to rapidly eliminate the re-infection or prevent the re-apparition of the cancer. That is why we wanted to use genes involved in HSC self-renewal to increase CD8 Tm cell self-renewal, particularly *Hoxb4* because it is a known factor capable of expanding HSCs without inducing leukemia. Our strategies did not show any advantage for CD8 Tm cell self-renewal. However, a great benefit could be found if the *Hoxb4* overexpressing CD8 Tm cells that stay in the host can better respond to a reencounter. We did not explore this potential with our experiments. For this, the *Listeria monocytogenes* model, where we can infect the transplanted mice and follow the response *in vivo* could give us valuable information. Finding a mechanism to promote a better secondary response could also be used as a strategy to increase the ability of the CD8 Tm cells to fight against recurrent pathogens or cancerous cells.

Conclusion

The objective of this study was to evaluate the competitive advantage provided by the overexpression of *Hoxb4* on CD8 Tm cells. We were looking particularly at the ability of this gene to enhance the self-renewal of the CD8 Tm cells. When we evaluated young and old *Hoxb4* overexpressing mice, T cell homeostasis was maintained as in normal mice which means that these cells are not proliferating more in normal conditions. We also found that the transgene does not affect the capacity to *in vitro* generate CD8 Tm cells. With the transplantations assays, we found that *Hoxb4* does not enhance CD8 Tm cells self-renewal neither in immunocompetent or immunodeficient mice. Comparing WT and *Hoxb4* overexpressing cells transplanted cells, we did not observe changes in the phenotype in any marker evaluated, the cells where capable to migrate to the different lymphoid organs but the ability to replace WT cells in normal host was not enhanced. Therefore, we conclude that *Hoxb4* overexpression does not have an impact on the self-renewal of CD8 Tm cells under these experimental settings.

Future plans

It would be interesting to identify novel factors involved in CD8 Tm self-renewal to improve their maintenance. A selection for candidate genes has already given us 45 candidate genes. We based our selection strategy on three different points: 1) The genes highly expressed in both CD8 Tm and HSC, these information was obtained from the public data base in the immunological genome project. 2) Their reported property to enhance HSC self-renewal (Deneault et al., 2009). 3) The transcriptional program that is shared by both cell types (Luckey et al., 2006). However, HSCs are not the only cells that need to self-renew therefore, comparing the behavior of our candidates in other cells like leukemic and neural stem cells could give us a hint on which could be the best candidates to explore. We believe that the cells could use similar mechanisms to be maintained and that is the reason why the best candidates would be involved in the different cell types.

We could first evaluate the expression of our 45 candidates in our *in vitro* generated Tm cells. A retroviral expression system with a Flag tag and GFP selection could be used to stably overexpress the candidate genes in the *in vitro* generated CD8 Tm cells at the third day of culture, validate by Western blot using anti Flag. The CD8 Tm development and changes in the number of cells generated would be evaluated to confirm any difference compare with the WT. Furthermore, to screen the intrinsic potential of these genes for self-renewal, cells could be cultured in the absence of IL-15, because IL-15 provides the necessary signal to promote CD8 Tm cell self-renewal division. Cells overexpressing the most promising genes could be finally evaluated *in vivo*. Competitive assays could be performed, which would be analysed for growth (cell numbers), cell cycle (BrdU), phenotype, apoptosis (AnnexinV/7-AAD), maintenance, homing and functionality over time.

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