Fluorescent in vivo models for hematopoietic stem cell and lymphoid lineage analysis

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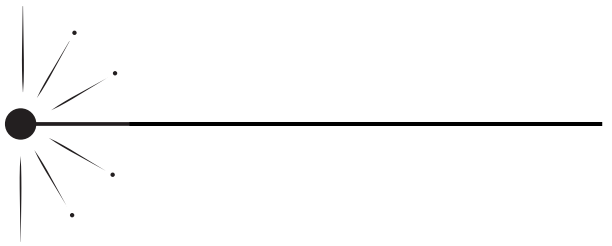
General discussion



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In this thesis, we investigated and created improved fluorescent protein murine models to study hematopoiesis. Even though a wide range of experimental murine models have given valuable information on hematopoietic stem cell dynamics and at which stages cell signaling affects self-renewal or cell differentiation patterns, insufficient effort has been invested in optimization or critical analysis whether those models were portraying meaningful results. Increasingly complex genetic barcoding cell tracking models are excluding highly valuable spatial information which a fluorescent protein cell tracking model can offer. Instead, the focus on quantitative data and increased cell marking has incremented experimental costs and reduced comprehensive interpretation of the contextual setting. On the other hand, cell signaling reporter models have been utilized conveniently without in most cases a critical understanding of either the genetic context or the reporter protein dynamics to properly measure ranges of cell signaling reporter levels, especially in fragile cells. Moreover, there are currently no existing multi-reporter models to study cell signaling cross-talk collectively, as current knowledge is based mostly on stand-alone, single pathway reporter models.

Hence, the aims of this thesis were; 1) to improve an existing murine cell tracing model to visually define hematopoietic stem and progenitor cell (HSPC) clonal outgrowth. We also intended to define fluorescent activated cell sorting (FACS) protocols to combine 10 fluorescent protein (FP) cell tagging with extracellular characterization; 2) to assess Wnt signaling activity in rare B-1 B cells with the use of a well-established Axin2-LacZ reporter model; 3) to create an easier to employ canonical Wnt signaling reporter model with a stable and reliable fluorescent protein for the measurement of low signaling levels in fragile cells; 4) to design a murine multi-fluorescent protein reporter model to measure signaling pathway crosstalk and integration. This included the understanding of expected signaling pathway combinations from previous reported literature and the best performing fluorescent protein combinations for reliable FACS and confocal imaging protocols.

In chapter 6, we discuss the obtained results and address the impact of our models on the understanding of hematological development from a broader perspective. We first will focus on existing cell tracking models and the applications, followed by the role of conserved signaling pathways in hematopoiesis which special attention to canonical Wnt signaling, as well as the clinical relevance of our models.

HEMATOPOIETIC CLONALITY AND DIFFERENTIATION

The understanding of hematopoietic stem cell (HSC) biology has been a challenging

endeavor with many different approaches and experimental models. Various gene expression/silencing models and reporter models reveal contradicting results and therefore demonstrate that hematopoiesis is a complex and niche dependent process. Much remains to be revealed, ranging from cell clone activity, division and differentiation rate, to unraveling which signals at which developmental stages are responsible for sustained hematopoiesis. Additionally, physiological and stress-hematopoiesis are essentially different and the niche environment affects the decision making of the hematopoietic cells. Combined, these factors emphasize the importance of choosing a representative experimental model for each research question.

Clonal cell tracking models

Transplantation models have been useful to understand hematopoietic development and have given the opportunity to characterize as well as quantify the cells responsible for hematopoietic recovery after bone marrow ablation. Within the HSPC compartment, several cells have been found to have stem cell potential or at least are responsible for the clonal outgrowth in the transplanted individual. However, much debate still remains about the frequency and exact identity of stem cell-like clones. According to limiting dilution assays, one single HSC could reconstitute long-term multilineage hematopoiesis in mice [1,2]. However, this does not reflect the natural function and heterogenicity of HSCs during normal life, ageing or disease. Instead, clonal cell tracking models began to gain popularity to study better the stem cell origin and differentiation steps contributing to hematopoiesis. Three approaches were mainly cited: 1) color-coding with fluorescent proteins [3-5]; 2) genetic marking (barcoding) through viral vector insertion [6-8]; and a combination of both [9-11]. Each strategy, or model, has its benefits and drawbacks, leading to a segregation within the research community on which model is the best choice to study HSC contribution. The main issue is the marking efficiency which depends on the total number of possible marking variations and the number of clonogenic cells to be marked. Color-coding models tend to have limitations in the number of unique markings, while quantitative genetic marking models are denounced to have either low resolution (underestimation) or an overestimation of stem cell counts. Moreover, the increasing number of unique markings attained by newer quantitative genetic marking models produces the complication of cumbersome protocols, increased detection limits and sequencing or mapping errors [12]. Apart from the technical constraints that the cell tracking approach generates, the main question is whether these models are able to reveal the full spectrum of scenarios of clonal dynamics of the hematopoietic system.

Stress hematopoiesis and post-transplantation recovery

The current dogma on clonal contribution for prolonged hematopoiesis is an unresolved debate between a reduced number of stable HSCs versus a larger number of progenitor cells. Normal blood production is polyclonal, meaning that after transplantation and even more so in a physiological setting, hundreds to thousands of HSPCs contribute to hematopoiesis [13]. However, an apprized distinction between stress hematopoiesis and physiological hematopoiesis is often neglected when comparing results of clonal cell tracking models. Even though understanding physiological hematopoiesis dynamics is interesting for fundamental knowledge purposes, it is the understanding of stress-hematopoiesis that is most imminent for clinical relevance.

The main initial purpose of the transplanted graft material is to evoke an early recovery from cell aplasia which is governed by the early reconstitution phase followed by the steady-state phase. Firstly, the short-lived MPPs drive recovery of the hematopoietic system which after exhaustion (a few months) is followed up by long-term HSCs (LT-HSCs). However, how the cellular differentiation dynamics take place in these reconstitution phases is still unclear. In murine mouse models, the leading concept is that transplanted HSCs are heterogenous, have heterogeneous reconstitution patterns and have a bias to either de lymphoid or myeloid cell fate [14-18]. This concept was opposed by Carrelha *et al*., with the finding of megakaryocyte/erythrocyte-restricted HSCs with long term survival [19]. Subsequently, a comprehensive study was performed taking into account the experimental set-up conditions which could affect HSC functionality. This study mainly confirms that hematopoietic differentiation occurs differently under stressed conditions such as transplantation, conditioning or immunodeficiency, compared to unperturbed hematopoiesis [20]. Most notably, organ damage due to the preconditioning treatments have a tremendous effect on the response of transplanted cells, which will be discussed further ahead. In fact, lineage-biased LT-HSCs were merely found in stress-prone and perturbed conditions such as irradiation and antagonistic anti-c-kit antibody (ACK2) preconditioning. The biggest differences were delineated to dominant HSC clone differentiation and lineage bias, which resulted into biased blood production [20]. Interestingly, irradiation-mediated transplantation led to an HSC expansion wave of unbiased HSC clones, apart from the two myeloid-biased expansions waves and one lymphoid expansion wave during lineage commitment [20]. In humans, similar lineage commitment pathways have been reported. Translational probabilistic models in transplanted patients based on the study of integration sites of a therapeutic vector as cell-tracing marker, underlines that even though MPPs are more active in the early phases of recovery, it is in the later steady state phase where primitive HSCs govern hematopoiesis [21-23]. Considering that these patients

were pre-conditioned with irradiation therapy, a similar population of unbiased HSC clones could be responsible for sustained hematopoiesis even though the possible therapeutic transgene effect should not be neglected. However, as bone marrow transplantation with LT- and/or ST-HSCs is the golden standard of HSC functional assays and most attention is directed to long-term reconstitution, the functional output and cell production of multipotent progenitor cells (MPPs) is seldomly studied and thus poorly understood.

In **chapter 1**, we adopted a ten-color cell marking mouse model by inducing differential combinatorial expression of four original colors to study stress hematopoiesis shortly after transplantation. The original mouse model was used to demonstrate spatio-temporal gut stem cell dynamics using four colors [24]. Our retroviral Cre strategy showed efficient transduction of Lin^- $c\text{-}kit^+$ (LK) cells, resulting in the expression of the maximum of ten color combinations. As the retroviral vector enters easier into dividing progenitor cells within the LK fraction, we were able to study the short-term cellular output and marking kinetics of these cells. Additionally, we proposed a multi-color FACS strategy to quantify the cell marking activity while still leaving margin for additional fluorescent cell characterization markers. Since this strategy leaves the cells untouched *in vivo*, our model is useful to study hematopoietic cell characterization. Moreover, genetic marking cell tracking models focus primarily on the quantification of cells, whereas information on functional heterogeneity and formal definition of stem cells is often omitted. Indeed the ten-color marking does not seem to be sufficient to study the full spectrum of HSC dynamics or at least is touching the proposed marking limits [12]. However, this model remains meaningful for low-complexity cell tracking such as early B- and T cell development. Furthermore, our model gives the opportunity to study transplantation stress hematopoiesis with spatial clues of the niche. For example, T cell progenitor cell tracking in the thymic niche would give interesting insights into T cell development. By being able to combine this low-complexity marking model with multiple additional extracellular characterization markers, our model is applicable for cellular definition as well as spatial information through confocal microscopy or even imaging flow cytometry [25].

CONSERVED CELLULAR SIGNALING PATHWAYS

Wnt signaling is a highly conserved pathway with a prominent role in adult stem cells. Dysregulated Wnt levels can disturb the development and function of various organs [26], and are also known to play a decisive role in a variety of malignant and nonmalignant hematologic diseases. According to the “just-right” signaling model, successful triggering of tumorigenesis is driven by impaired signal transduction at a specific level [27,28]. A too low

signal will not provide enough transcriptional response, while a too high signaling activity might trigger an apoptotic response [29]. Especially for Wnt signaling several studies have indicated that distinct Apc/β-catenin levels affect stem cell differentiation [30]. These dosage levels are thought to be regulated through gene-dosage and/or protein-dosage which can drive regional of localized β-catenin expression patterns. In fact, these distinct β-catenin levels could lead to not only to activate, but also repress Wnt target genes [31]. The Wnt dosage model proposes that the exact right amount of Wnt signaling is required for correct HSC cell functioning [32,33] and the same kinetics is generally true for all signaling pathways and cell types. Especially when addressing signaling pathways, one must contemplate that it is not simply a dichotomous "on" or "off" state. Instead, it is a balanced reaction to various interceding proteins which results into dosage-driven effects. This means that conserved signaling pathways could be considered as therapeutic targets to exert controlled manipulation for a beneficial outcome.

Indeed, Notch, TGF-β/BMP and Hedgehog signaling are thought to play important roles in physiological and stress hematopoiesis [34]. For example, bone marrow damage caused by chemotherapy induces fundamental changes in the expression of Notch ligands Delta-like 4 (DLL4) and DLL1 of the vascular endothelium which issues HSPC myeloid skewing instead of common lymphoid progenitor (CLP) T-lineage specification [35]. In fact, sex steroid hormone ablation therapy has shown to increase DLL4 expression and cause improved HSC self-renewal and lymphoid differentiation [36].

Interestingly, osteo Wnt5a and perivascular BMP4 were upregulated after the stress hematopoiesis insult [35]. The non-canonical Wnt5a was previously reported to be increased in short-term HSC repopulation by maintaining HSCs in a quiescent G0 state [37]. However, canonical Wnt signaling is necessary to activate HSCs and induce self-renewal and differentiation [38]. A similar phenomenon is known in aged-HSCs, where Wnt5a-driven non-canonical Wnt signaling led to a decreased lymphoid output and increased myeloid output [39,40]. Conversely, canonical Wnt3a overexpression led to increased B lymphopoiesis and decreased myeloid cells after transplantation [41], indicating that Wnt signaling affects cell fate decisions in different manners. Expansion of HSCs for transplantation with Wnt modulating cytokines or other signals have only been partially successful, mainly due to the lack of control of the Wnt dosage kinetics [32]. Our canonical Wnt reporter model in **chapter 3** is an interesting model to measure and understand the dosage kinetics of these therapeutic agents to improve *ex vivo* HSC expansion protocols.
BMP4 on the other hand is an interesting therapeutic target for thymic regeneration after damage as it has a prominent role during thymus organogenesis [42]. Importantly, *ex vivo*

expanded thymic endothelial cells delivering BMP4 improved thymic reconstitution after adoptive transfer [43]. BMP4 was found to bind to receptors on thymus epithelial cells (TECs) and stimulated the upregulation of *FoxN1* and its target genes [44]. Interestingly, *FoxN1* is a known Wnt target gene, indicating anew the implication of Wnt signaling for regulating the thymic niche [45,46].

The other member of the TGF-β superfamily, the TGF-β pathway is thought have implications in hematopoiesis as well. Although there seems to be indication of a possible therapeutic agent for thymus rejuvenation, the role of this pathway is poorly understood in T cell development and thymic function [42]. Therefore, most interest is concerning the control of HSCs, particularly in quiescence and self-renewal through the regulation of cell cycle kinases [47]. In fact, *in vivo* temporal inhibition of TGF-β in HSCs resulted in enhanced regeneration post transplantation [48], indicating a possible therapeutic avenue that can aid in patient recovery before the HSCs return to quiescence. However, the multifaceted nature of TGF-β and its complex mechanisms (including pathway cross-talk), argue that more knowledge is required through reporter models, such as the proposed PAI-1-mScarlet-I TGF-β proposed in **chapter 5**. Indeed, TGF-β biphasic response (hinting towards a dosage mechanism) and varying TGF-β sensitivity have been proposed, which makes it interesting to investigate further [47]. As TGF-β ligands are largely expressed by megakaryocytes and deposited in the bone matrix, the mScarlet-I fluorescent protein is ideal for deep-tissue signaling to understand the function of TGF-β in the bone marrow niche.

Finally, the role of Hedgehog (Hh) signaling in hematopoiesis is less understood than the other pathways. Generally, Hh is accepted to be crucial for correct embryonic development and HSC development [49], however its role seems to be most prominent during altered signaling [50]. Trowbridge *et al.,* proposed that the Hh downstream signaling pathway is involved in HSC quiescence through cell cycle regulation, for continuous activation of Hh exhausts HSCs [51]. Moreover, Hh ligands expressed by bone marrow stromal cells are thought to act as tumor survival signals and support tumor growth in lymphoid and myeloid malignancies such as acute myeloid leukemia (AML), chronic myeloid leukemia (CML) and acute lymphoblastic leukemia (ALL). Therefore, several therapeutic strategies have been proposed to block Hh signaling, but have not been very promising [50]. As the Hh pathway is known to cross-talk with other pathways such as Notch and Wnt signaling, its exclusive role is hard to determine or understand. The Hh Gli1-nNeonGreen reporter model proposed in **chapter 5** could be of interest to clarify its contribution in stem cell maintenance and self-renewal capacity and even a combination of reporter models could

be made to study pathway cross-talk interaction [52].

In sum, conserved signaling pathways play intricate roles in diverse stages of hematopoiesis with varying signaling levels and complex intra-pathway regulation mechanisms. Stand-alone reporter models for each unique pathway would improve knowledge about dosage-driven homeostasis and stress-hematopoiesis mechanisms. Yet it is the combination of several reporters which will give truly insightful information on how to regulate hematopoiesis readily for clinical application.

WNT SIGNALING IN LYMPHOPOIESIS

The evolutionary conserved Wnt signaling is crucial in many developmental processes as well as in the maintenance of adult tissue homeostasis. The significant impact of aberrant canonical Wnt signaling has led to substantial efforts to develop therapeutic strategies to target this pathway [26]. However, its role is versatile; involving several signaling proteins, varying signal strength or even cross-talk with other signaling pathways. For example, canonical Wnt signaling is important for both T and conventional B cell development, however the involved transcription factors are different. Studies in mice deficient for these transcription factors showed that the *Lymphoid enhancer factor* (*Lef1*) is crucial for B cell development [53,54] as *T cell factor* (*Tcf1*), and to some extent, *Lef1* is for T cell development [55,56]. Indeed, along the developmental stages of distinct HSC cell subsets, the signaling mechanism and signal strength is known to vary [57,58]. Most notably, it is in the T cell subsets where canonical Wnt signaling is most active [33] as for conventional B cells it is mostly important in the early developmental stages [33,59,60].

B-lymphopoiesis

In **chapter 2**, we studied the role of canonical Wnt signaling in B-1 B cells. B-1 B cells can be subdivided into B-1a cells and B-1b cells and their origin is believed to be distinct. As B-1a cells are considered to originate primarily from the fetal liver, Wnt3a could be of interest for their stem cell-like self-renewal capabilities. Wnt3a was shown to be essential for self-renewal of fetal liver HSPCs [61]. Furthermore, Wnt3a also plays a role in conventional pro-B-cell proliferation *in vitro* [54]. Lastly, Wnt3a is expressed in the bone marrow niche, therefore canonical Wnt signaling could influence the highly disputed HSC-derived B-1 a cell generation. We have shown that murine B-1 progenitors in the bone marrow express canonical Wnt signaling and that high expression of Wnt3A leads to B-1a generation. This means that, similar to HSCs [33], canonical Wnt signaling has a differentiation effect on B-1 progenitors. However, Luis *et al*., unraveled that the dosage of active Wnt signaling is deterministic for the cellular response. Mild canonical Wnt signal

led to increased self-renewal, whereas too high Wnt signaling led to a block of HSC multilineage reconstitution [32,33]. Although for conventional B-2 cells it was shown to be ineffective [33], this dosage concept would be of interest to study in B-1 cell development with the more sensitive Axin2-mTurquoise2 reporter proposed in **chapter 3**. Apart from its convenience to measure canonical Wnt signaling in the scarce B-1 (progenitor) cells, it could also give a clearer picture on fluctuating reporter levels to unravel the ontogeny enigma of the B-1 subpopulations.

T-lymphopoiesis

In humans and rodents, canonical Wnt is expressed in the thymus (https://www.ncbi.nlm.nih.gov/gene/12006), indicating that Wnt signaling might be important for T cell development. Indeed, the Axin2-LacZ reporter has demonstrated oscillating canonical Wnt signaling levels in each T cell subset [33]. Moreover, precisely controlled canonical Wnt signaling is important for thymus morphogenesis and supports normal thymic epithelial cell differentiation although deregulation does not negatively affect thymic function [45,62]. However, little functional information is known about canonical Wnt signaling in the thymocytes during the different developmental stages due to the difficulties of measuring the narrow signaling ranges. Our protocol in **chapter 4**, has granted the possibility to measure small Wnt signaling differences in fragile cells such as thymocytes. In combination with the cytoplasmic and nuclear localization of β-catenin, the dynamics of canonical Wnt signaling can be analyzed with precision.

Based on our preliminary data to study the Wnt dosage effects in our Axin2-mTurquois2 homozygote reporter **(chapter 3)**, we show that in an Axin2 knock-out (Axin2-mTurquoise2 Tg/Tg) early T cell development is partially blocked after transplantation in healthy individuals **(figure 1A)**. On the other hand, the Axin2-mTurquoise2 Tg/0 thymocytes show to have enhanced T cell development compared to the wildtype competitor cells, although this effect was not visible at the DN1 stage. Interestingly, Luis *et al.,* demonstrated a similar canonical Wnt dosage effect in thymocytes [33]. However, the Lck-Cre mediated Wnt expression in *Apc*-mutant lines only gave the possibility of altering the Wnt signaling from the DN3 stage and onwards, for which our preliminary data shows the first results of Wnt dosage effects in early T cells. Unfortunately, it is not possible to quantify the Wnt dosage level via Axin2 expression for comparison to the dosages of Tiago *et al.,* as the Axin2-mTurquoise model has an incomplete *Axin2* gene and disrupted Axin2 protein due to the genetic insertion strategy **(chapter 3)**. Instead, we have measured the ratio of nuclear/β-catenin cytoplasmic expression in DP thymocytes, showing that indeed there is progressively more canonical Wnt expression when the *Axin2* gene is disrupted

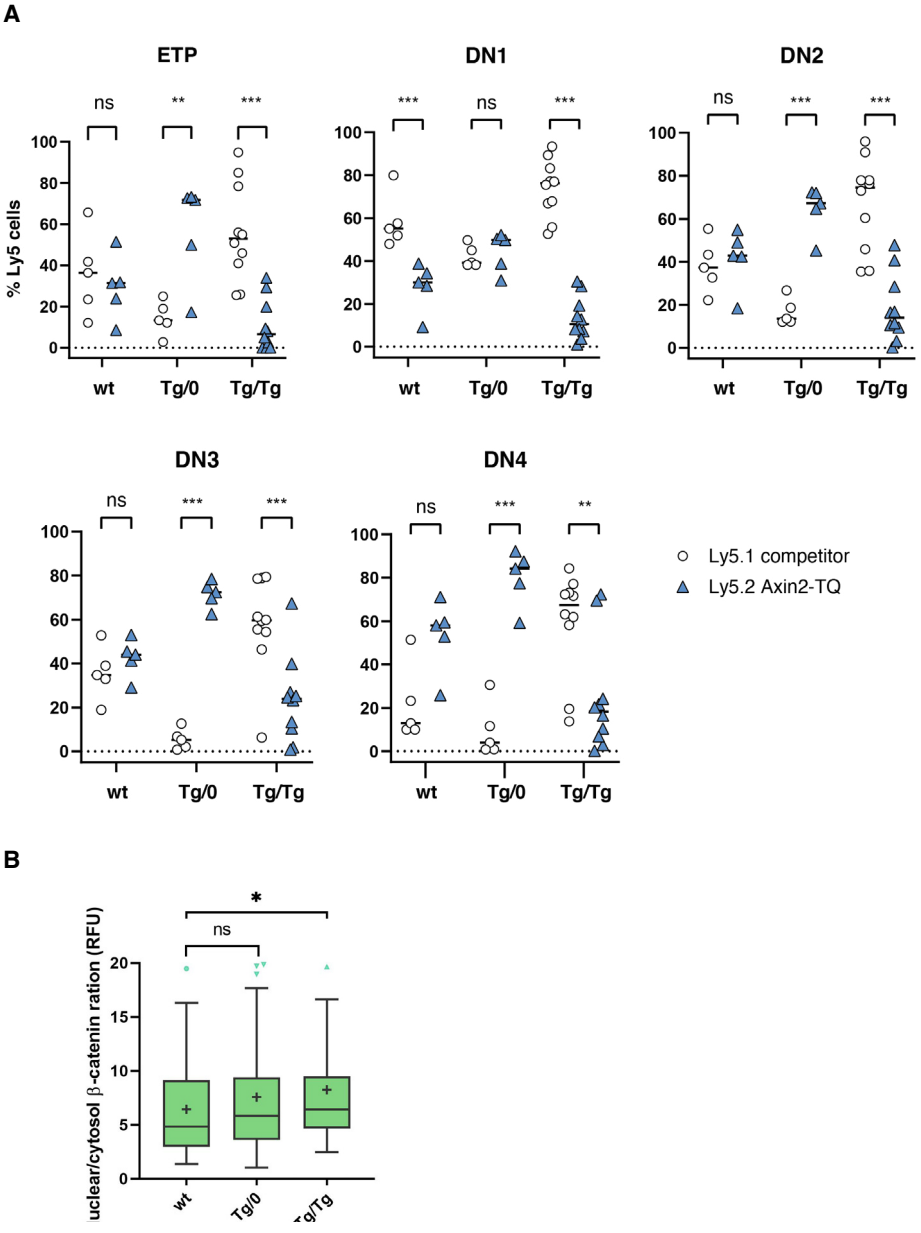

Figure 1: Axin2-mTurquoise reporter model in T cell development. (A) *T cell development stages after primary competitive transplantation of Ly5.2 Axin2-mTurquoise2 Lin⁻Sca-1⁺c-Kit+ (LCK) HSCs and Ly5.1 wild type HSCs in healthy C57BL/6 donor mice.* ***(B)*** *Nuclear/cytoplasmic total β-catenin expression in Axin2-mTurquoise2 pan-thymocytes per genotype. Bargraph represent the median, whereas the + represents the mean.*
*Non significant (ns), *p<0,05, **p<0,01 and ***p<0,001 when indicated.*

(figure 1B). The role of canonical Wnt signaling in early T cells could be related to T cell fidelity, apart from a previously reporter proliferative function [57]. Garcia-Perez *et al.,* showed that TCF1 expression in early thymocytes is primarily driven by Notch and acts as a transcriptional activator of *Gata3* and *Bcl11b* [63]. However, functional data should support this notion through the measurement of β-catenin and binding to the TCF1 transcription factor.

CLINICAL RELEVANCE

Animal models have been crucial for biomedical research for the reason that it is often not possible to conduct such studies in humans. The genetic insertion of fluorescent proteins into human cells as has been performed or proposed in the clonal cell tracking model in **chapter 1** and the cell signaling models in **chapter 3 and 5**, is not permitted due to ethical reasons. From a practical perspective, the employed techniques are equally applicable for any cell type of any species, however the effect of implementing an artificial protein into a human cell is till date not attempted nor its benefit justifies the risk. Therefore, the clinical relevance of the proposed models in this thesis regards a translational character.

Ageing

Ageing and the increasing life expectancy are causing major health and economic issues. With advancing age, there is a decline in the efficiency and fidelity of homeostasis and the repair processes in the body. This is thought to be caused by a decline of stem cell populations as well as a decline in their effectiveness [64]. Lymphopenia is one of the effects which is seen in an ageing population, however cancer is another prominent disease starting at the age of 50.

Furthermore, the increasing execution of hematopoietic stem cell transplantations (HSCTs), leads to an inevitable increase in lymphopenic patients as well. Therefore, there is considerable interest to evaluate T and B cell quantity and quality to guide treatment directions or even identify biomarkers to monitor immune responses [65]. Accurate and easy to apply reporter models as proposed in **chapter 3** and **chapter 5** are interesting tools to test new therapeutic agents and could possibly aid in the definition of required therapy to improve lymphocyte output.

Hematopoietic stem cell transplantations

Hematopoietic stem cell transplantations (HSCTs) are an effective method for treating malignant, non-malignant and inherited genetic disorders [66,67]. Applications are related

to hematopoietic disorders such as immune dysregulation, refractory autoimmunity, cytopenia or hematopoietic malignancies in adults [68] and refractive lymphoma, leukemia, metabolic or autoimmune diseases to solid tumors and central nervous system tumors in pediatric patients [69]. The understanding of hematological recovery after transplantation, could improve transplantation protocols or even lower the considerably high mortality rate [70]. The main problems causing patient fatalities are: infections, organ damage, cancer relapse and severe immune reactions, however uncertainty about HSC engraftment after myeloablative treatment is another concerning factor which is currently out of our control. Myeloablative therapy is comprised out of a high dose of chemotherapy and/or radiation treatment which is essential to: 1) make room in the bone marrow for the transplant/ graft, 2) suppress the patients' immune system to reduce the risk of graft rejection and 3) destroy any remaining cancer cells of the patient. It is in fact this same pre-conditioning treatment that causes organ damage, such as in the bone marrow and the thymus leading to dysfunctional CLP T cell lineage skewing, impaired B lymphopoiesis and improper T cell development [71]. The decrease in T cell development can lead to long-term suppression of thymus function and accelerate age-associated thymic involution [72].

Lymphopenia

Immune dysfunction is a recurring problem in lymphopenic patients which is regularly caused by hematopoietic stress or injury and is especially life-threatening in HSCT patients. Complete immune reconstitution can take several months to years depending on the cell type and HSCT. T and B lymphocytes are the slowest immune cells to reconstitute, taking more than two years to fully recover in transplants [73,74]. In particular T cells are sensitive to negative insults caused by cytoablative therapy, infections, graft-versus-host disease or even age-related thymic involution. A declined T cell output and T cell senescence with restricted T cell receptor (TCR) repertoire has detrimental implications in future immune responses and overall adverse clinical outcome [75,76]. Even though numerical T cell reconstitution can be achieved by IL7 and IL15 treatment, it is the thymic niche environment which is believed to be an important contributor for recovery of a diverse and functional T cell pool via *de novo* T cell generation [77]. The crosstalk between bone marrow-derived T cell progenitors and the thymic stroma are responsible for the T cell development process [78], making this interaction notably interesting. Certainly, several strategies have entered into clinical trials to enhance immune recovery [77]. These cytokines and growth factors are secreted by epithelial and mesenchymal stromal cells, however also a few hormones and hormone-like mediators have strong influence on the T cell numbers. Nonetheless, the effects are either not generating *de novo* T cells in older patients or the effects are transient or even cause deleterious effects when combined with

other therapies. In sum, several approaches are being explored however several barriers; such as age, defective stromal microenvironment and reduced response to homeostatic cytokines that limit immune regeneration must be overcome conjointly [77].

With age, progressively more HSCs have a reduced homing and engraftment capacity [79]. This leads to a significant reduction of intrathymic lymphoid progenitors contributing to the T cell periphery output [80]. The decline in thymus function results in thymic involution which is another key contributor to decreased thymic function and progressive decline of immune functionality with age [81]. As the thymus involutes, the naïve T cell pool is maintained by homeostatic proliferation [82,83]. Interestingly, thymic atrophy has a different onset between mouse and human. Whereas in mice, the thymus undergoes a reduction in volume with age, the human thymus remains almost unchanged in size and instead has a perturbed microenvironment of the stromal cells [84] with loss of thymic epithelium and increase of fat. Taking into account that most HSC transplantations occur in older patients, the fact of a suboptimal functioning thymus worsens the clinical outcome further on top of the cytoablative-induced damage. Indeed, there is an inverse relationship between transplant recipients age and T cell recovery after transplantation [85]. Age directly correlates with an increased risk of opportunistic infections, leukemia relapse and adverse clinical outcome [73].

In **chapter 1**, we recommend the use of the fluorescent cell tracking model to visualize clonal interaction within the thymus to study the aforementioned phenomena. The use of the murine model can give insights into spatio-temporal *de novo* T cell recovery occurs while being able to test different stem cell sources or *ex vivo* stem cell preconditioning regiments. All the more considering the difficulty of studying the effects of the aforementioned immune recovery strategies, cell-based approaches might give prompt answers instead of lengthy *in vivo* models. Organoids or scaffolds are recent advances that could be combined with the fluorescent cell tracing model from **chapter 1** or the vector-based color barcoding model as employed in the humanized NOD/SCID (NSG) xenograft [9].

Delay in B cell reconstitution is another problem for HSCT patients increasing the risk for infection and the development of graft-versus-host disease [86,87]. Even though immunoglobins from patient plasma cells survive pre-transplantation conditioning or donor-derived mature B cells can be administered [88], just like T cells, the B cell restoration is primarily mediated by *de novo* generation from bone marrow progenitors [89]. However, the delayed recovery of CD4 T cells impairs B cell differentiation, arrests maturation and functional reconstitution, leading to a decreased response to vaccines [86,90].

Comparable to thymic atrophy, both conventional B-2 B cell and B-1 B cell frequencies decrease with advancing age [91,92], more prominently in humans than in mice. Functionally, this decrease and changed proportion of B cells, impacts the antibody response to vaccination [93,94]. The age-related loss of diversity in the B cell repertoire generates antibodies which are less protective compared to younger individuals, which starts to be significant from the age of 50 [92,95,96]. What the exact role is of B-1 cells and how they recover after a lymphopenia is still unclear. In **chapter 2**, we have shown the role of Wnt signaling in the self-renewal capacity of B-1 cells in relatively young mice. However, it would be interesting to study the role of Wnt signaling after bone marrow transplantation of older HSCs with the Axin2-mTurquoise2 model in **chapter 3**. As the morphology changes with advanced age in B-1 cells which results into a high nucleus to cytoplasm ratio [92], the protocol in **chapter 4** will be valuable to measure the low canonical Wnt signaling through the reporter activity and cytoplasmic versus nuclear β-catenin expression.

Allogeneic HSCT patients have a high incidence of infectious diseases which causes up to 25% of all deaths after transplant [97]. Interestingly, B-1 B cells which offer a strong humoral immunity against pneumonia [98], influenza [99] and other infectious diseases. This is especially interesting to cover the lag time of T and B cell recovery in immune-compromised patients. However, little data is known about B-1 B cell recovery after HSCT. Partially because it has been difficult to discriminate between B-2 and B-1 B cells in humans. Quach *et al.*, reported that B-1 cells were found in the circulation of mobilized HSC transplanted patients at 8 weeks post transplantation. However, no distinction was made for B-1a and B-1b subsets, as it is claimed that human B-1 B cells display characteristics of both subsets. In rodents nonetheless, B-1 B cells are distinguished by B-1a and B-1b subsets which are thought to have disparate ontogeny [100-102], even though others claim that both subsets can be generated from HSCs [103-105]. Yet, human bone marrow and cord blood $Lin^{-}CD34^{+}CD38^{lo}$ stem cells have shown to generate both B-1 and B-2 lineages when transplanted into neonates of immune-deficient NSG mice [106]. The authors also note that unidentified committed B-1 and B-2 progenitor subsets in the transplanted graft are probable due to the lack of identifying markers, meaning that they still might disparate in origin. Alternatively, Ghosn *et al.*, showed that B-1a B cells cannot be reconstituted from human LT-HSCs, implying that HSCT patients have a shortcoming immune system with the lack of these B-1a cells [107]. Summarizing these contradicting theories and results, poses the question how B-1 cell commitment is effectuated. In **chapter 2**, we have shown that high expression of Wnt3A led to B-1a generation from bone marrow B-1 progenitors. This means that even though the initial ontogeny of B-1a cells might be from fetal liver, also HSC-derived B-1a cells can be generated. It would be interesting to study

whether the same B-1a development is influenced by Wnt3a with the Axin2-mTurquoise2 reporter construct (**chapter 3**) into human $Lin^{-}CD34^{+}CD38^{lo}$ stem cells. Additionally, the reporter activity patterns between the B-1 and B-2 B cell subsets and subsequent B cell development could give interesting hints for clinical application after HSCT.

CONCLUDING REMARKS

In this thesis we have implemented new experimental mouse models and showed how fluorescent proteins can be useful as reporters to understand hematopoietic development. The purpose of this is not only to obtain fundamental knowledge but also to develop tools to support clinically relevant questions involving HSCT and therapeutic strategies.

As also discussed in **chapter 5**, a future promising field of personalized research will be to use similar reporter models in human cells directly instead of in rodent animal models. The CRISPR-Cas9 technique as utilized in **chapter 3**, is easily applicable in human cells which can be either primary material or cell lines. In combination with bioengineered mini-organs or even mini-organs on a chip, a direct read-out of the natural 3D context can be studied considering the niche environment, which is highly instrumental when interpreting clinical outcome/results.

REFERENCES

1 Osawa, M., Hanada, K., Hamada, H. & Nakauchi, H. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. *Science* **273**, 242-245, doi:10.1126/science.273.5272.242 (1996).

2 Notta, F. *et al.* Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. *Science* **333**, 218-221, doi:10.1126/science.1201219 (2011).

3 Yu, V. W. C. *et al.* Epigenetic Memory Underlies Cell-Autonomous Heterogeneous Behavior of Hematopoietic Stem Cells. *Cell* **168**, 944-945, doi:10.1016/j.cell.2017.02.010 (2017).

4 Ganuza, M. *et al.* Lifelong haematopoiesis is established by hundreds of precursors throughout mammalian ontogeny. *Nat Cell Biol* **19**, 1153-1163, doi:10.1038/ncb3607 (2017).

5 Martinez, R. J., Neeld, D. K. & Evavold, B. D. Identification of T cell clones without the need for sequencing. *J Immunol Methods* **424**, 28-31, doi:10.1016/j.jim.2015.04.018 (2015).

6 Teets, E. M., Gregory, C., Shaffer, J., Blachly, J. S. & Blaser, B. W. Quantifying Hematopoietic Stem Cell Clonal Diversity by Selecting Informative Amplicon Barcodes. *Sci Rep* **10**, 2153, doi:10.1038/s41598-020-59119-8 (2020).

7 Pei, W. *et al.* Polylox barcoding reveals haematopoietic stem cell fates realized in vivo. *Nature* **548**, 456-460, doi:10.1038/nature23653 (2017).

8 Sun, J. *et al.* Clonal dynamics of native haematopoiesis. *Nature* **514**, 322-327, doi:10.1038/nature13824 (2014).

9 Brugman, M. H. *et al.* Development of a diverse human T-cell repertoire despite stringent restriction of hematopoietic clonality in the thymus. *Proc Natl Acad Sci U S A* **112**, E6020-6027, doi:10.1073/pnas.1519118112 (2015).

10 Gerrits, A. *et al.* Cellular barcoding tool for clonal analysis in the hematopoietic system. *Blood* **115**, 2610-2618, doi:10.1182/blood-2009-06-229757 (2010).

11 Yabe, I. M. *et al.* Barcoding of Macaque Hematopoietic Stem and Progenitor Cells: A Robust Platform to Assess Vector Genotoxicity. *Mol Ther Methods Clin Dev* **11**, 143-154, doi:10.1016/j.omtm.2018.10.009 (2018).

12 Bystrykh, L. V., Verovskaya, E., Zwart, E., Broekhuis, M. & de Haan, G. Counting stem cells: methodological constraints. *Nat Methods* **9**, 567-574, doi:10.1038/nmeth.2043 (2012).

13 Verovskaya, E. *et al.* Heterogeneity of young and aged murine hematopoietic stem cells revealed by quantitative clonal analysis using cellular barcoding. *Blood* **122**, 523-532, doi:10.1182/blood-2013-01-481135 (2013).

14 Busch, K. *et al.* Fundamental properties of unperturbed haematopoiesis from stem cells in vivo. *Nature* **518**, 542-546, doi:10.1038/nature14242 (2015).

15 Müller-Sieburg, C. E., Cho, R. H., Thoman, M., Adkins, B. & Sieburg, H. B. Deterministic

regulation of hematopoietic stem cell self-renewal and differentiation. *Blood* **100**, 1302-1309 (2002).

16 Sieburg, H. B. *et al.* The hematopoietic stem compartment consists of a limited number of discrete stem cell subsets. *Blood* **107**, 2311-2316, doi:10.1182/blood-2005-07-2970 (2006).

17 Lu, R., Neff, N. F., Quake, S. R. & Weissman, I. L. Tracking single hematopoietic stem cells in vivo using high-throughput sequencing in conjunction with viral genetic barcoding. *Nat Biotechnol* **29**, 928-933, doi:10.1038/nbt.1977 (2011).

18 Yamamoto, R. *et al.* Clonal analysis unveils self-renewing lineage-restricted progenitors generated directly from hematopoietic stem cells. *Cell* **154**, 1112-1126, doi:10.1016/j.cell.2013.08.007 (2013).

19 Carrelha, J. *et al.* Hierarchically related lineage-restricted fates of multipotent haematopoietic stem cells. *Nature* **554**, 106-111, doi:10.1038/nature25455 (2018).

20 Lu, R., Czechowicz, A., Seita, J., Jiang, D. & Weissman, I. L. Clonal-level lineage commitment pathways of hematopoietic stem cells in vivo. *Proc Natl Acad Sci U S A* **116**, 1447-1456, doi:10.1073/pnas.1801480116 (2019).

21 Cartier, N. *et al.* Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. *Science* **326**, 818-823, doi:10.1126/science.1171242 (2009).

22 Biasco, L. *et al.* In Vivo Tracking of Human Hematopoiesis Reveals Patterns of Clonal Dynamics during Early and Steady-State Reconstitution Phases. *Cell Stem Cell* **19**, 107-119, doi:10.1016/j.stem.2016.04.016 (2016).

23 Scala, S. *et al.* Dynamics of genetically engineered hematopoietic stem and progenitor cells after autologous transplantation in humans. *Nat Med* **24**, 1683-1690, doi:10.1038/s41591-018-0195-3 (2018).

24 Snippert, H. J. *et al.* Intestinal crypt homeostasis results from neutral competition between symmetrically dividing Lgr5 stem cells. *Cell* **143**, 134-144, doi:10.1016/j.cell.2010.09.016 (2010).

25 Hagert, C. F., Bohn, A. B., Wittenborn, T. R. & Degn, S. E. Seeing the Confetti Colors in a New Light Utilizing Flow Cytometry and Imaging Flow Cytometry. *Cytometry A* **97**, 811-823, doi:10.1002/cyto.a.24032 (2020).

26 Kahn, M. Can we safely target the WNT pathway? *Nat Rev Drug Discov* **13**, 513-532, doi:10.1038/nrd4233 (2014).

27 Albuquerque, C. *et al.* The 'just-right' signaling model: APC somatic mutations are selected based on a specific level of activation of the beta-catenin signaling cascade. *Hum Mol Genet* **11**, 1549-1560, doi:10.1093/hmg/11.13.1549 (2002).

28 Fodde, R. & Smits, R. Cancer biology. A matter of dosage. *Science* **298**, 761-763, doi:10.1126/science.1077707 (2002).

29 Kim, K., Pang, K. M., Evans, M. & Hay, E. D. Overexpression of beta-catenin induces apoptosis independent of its transactivation function with LEF-1 or the involvement of major G1 cell cycle regulators. *Mol Biol Cell* **11**, 3509-3523, doi:10.1091/mbc.11.10.3509 (2000).

30 Gaspar, C. & Fodde, R. APC dosage effects in tumorigenesis and stem cell differentiation. *Int J Dev Biol* **48**, 377-386, doi:10.1387/ijdb.041807cg (2004).

31 Kielman, M. F. *et al.* Apc modulates embryonic stem-cell differentiation by controlling the dosage of beta-catenin signaling. *Nat Genet* **32**, 594-605, doi:10.1038/ng1045 (2002).

32 Staal, F. J., Chhatta, A. & Mikkers, H. Caught in a Wnt storm: Complexities of Wnt signaling in hematopoiesis. *Exp Hematol* **44**, 451-457, doi:10.1016/j.exphem.2016.03.004 (2016).

33 Luis, T. C. *et al.* Canonical wnt signaling regulates hematopoiesis in a dosage-dependent fashion. *Cell Stem Cell* **9**, 345-356, doi:10.1016/j.stem.2011.07.017 (2011).

34 Zhang, J. *et al.* Identification of the haematopoietic stem cell niche and control of the niche size. *Nature* **425**, 836-841, doi:10.1038/nature02041 (2003).

35 Tikhonova, A. N. *et al.* The bone marrow microenvironment at single-cell resolution. *Nature* **569**, 222-228, doi:10.1038/s41586-019-1104-8 (2019).

36 Khong, D. M. *et al.* Enhanced hematopoietic stem cell function mediates immune regeneration following sex steroid blockade. *Stem Cell Reports* **4**, 445-458, doi:10.1016/j.stemcr.2015.01.018 (2015).

37 Nemeth, M. J., Topol, L., Anderson, S. M., Yang, Y. & Bodine, D. M. Wnt5a inhibits canonical Wnt signaling in hematopoietic stem cells and enhances repopulation. *Proc Natl Acad Sci U S A* **104**, 15436-15441, doi:10.1073/pnas.0704747104 (2007).

38 Sugimura, R. *et al.* Noncanonical Wnt signaling maintains hematopoietic stem cells in the niche. *Cell* **150**, 351-365, doi:10.1016/j.cell.2012.05.041 (2012).

39 Florian, M. C. *et al.* A canonical to non-canonical Wnt signalling switch in haematopoietic stem-cell ageing. *Nature* **503**, 392-396, doi:10.1038/nature12631 (2013).

40 Geiger, H., de Haan, G. & Florian, M. C. The ageing haematopoietic stem cell compartment. *Nat Rev Immunol* **13**, 376-389, doi:10.1038/nri3433 (2013).

41 Famili, F. *et al.* Discrete roles of canonical and non-canonical Wnt signaling in hematopoiesis and lymphopoiesis. *Cell Death Dis* **6**, e1981, doi:10.1038/cddis.2015.326 (2015).

42 Jurberg, A. D., Vasconcelos-Fontes, L. & Cotta-de-Almeida, V. A Tale from TGF-β Superfamily for Thymus Ontogeny and Function. *Front Immunol* **6**, 442, doi:10.3389/fimmu.2015.00442 (2015).

43 Wertheimer, T. *et al.* Production of BMP4 by endothelial cells is crucial for endogenous thymic regeneration. *Sci Immunol* **3**, doi:10.1126/sciimmunol.aal2736 (2018).

44 Tsai, P. T., Lee, R. A. & Wu, H. BMP4 acts upstream of FGF in modulating thymic stroma and regulating thymopoiesis. *Blood* **102**, 3947-3953, doi:10.1182/blood-2003-05-1657

(2003).

45 Balciunaite, G. *et al.* Wnt glycoproteins regulate the expression of FoxN1, the gene defective in nude mice. *Nat Immunol* **3**, 1102-1108, doi:10.1038/ni850 (2002).

46 Montero-Herradón, S. & Zapata, A. G. Delayed maturation of thymic epithelium in mice with specific deletion of β-catenin gene in FoxN1 positive cells. *Histochem Cell Biol*, doi:10.1007/s00418-021-02012-w (2021).

47 Blank, U. & Karlsson, S. TGF-β signaling in the control of hematopoietic stem cells. *Blood* **125**, 3542-3550, doi:10.1182/blood-2014-12-618090 (2015).

48 Brenet, F., Kermani, P., Spektor, R., Rafii, S. & Scandura, J. M. TGFβ restores hematopoietic homeostasis after myelosuppressive chemotherapy. *J Exp Med* **210**, 623-639, doi:10.1084/jem.20121610 (2013).

49 Heidel, F. H., Arreba-Tutusaus, P., Armstrong, S. A. & Fischer, T. Evolutionarily conserved signaling pathways: acting in the shadows of acute myelogenous leukemia's genetic diversity. *Clin Cancer Res* **21**, 240-248, doi:10.1158/1078-0432.Ccr-14-1436 (2015).

50 Lainez-González, D., Serrano-López, J. & Alonso-Domínguez, J. M. Understanding the Hedgehog Signaling Pathway in Acute Myeloid Leukemia Stem Cells: A Necessary Step toward a Cure. *Biology (Basel)* **10**, doi:10.3390/biology10040255 (2021).

51 Trowbridge, J. J., Scott, M. P. & Bhatia, M. Hedgehog modulates cell cycle regulators in stem cells to control hematopoietic regeneration. *Proc Natl Acad Sci U S A* **103**, 14134-14139, doi:10.1073/pnas.0604568103 (2006).

52 Chatterjee, S. & Sil, P. C. Targeting the crosstalks of Wnt pathway with Hedgehog and Notch for cancer therapy. *Pharmacol Res* **142**, 251-261, doi:10.1016/j.phrs.2019.02.027 (2019).

53 Galceran, J., Hsu, S. C. & Grosschedl, R. Rescue of a Wnt mutation by an activated form of LEF-1: regulation of maintenance but not initiation of Brachyury expression. *Proc Natl Acad Sci U S A* **98**, 8668-8673, doi:10.1073/pnas.151258098 (2001).

54 Reya, T. *et al.* Wnt signaling regulates B lymphocyte proliferation through a LEF-1 dependent mechanism. *Immunity* **13**, 15-24, doi:10.1016/s1074-7613(00)00004-2 (2000).

55 Tiemessen, M. M. *et al.* The nuclear effector of Wnt-signaling, Tcf1, functions as a T-cell-specific tumor suppressor for development of lymphomas. *PLoS Biol* **10**, e1001430, doi:10.1371/journal.pbio.1001430 (2012).

56 Verbeek, S. *et al.* An HMG-box-containing T-cell factor required for thymocyte differentiation. *Nature* **374**, 70-74, doi:10.1038/374070a0 (1995).

57 Ma, J., Wang, R., Fang, X. & Sun, Z. β-catenin/TCF-1 pathway in T cell development and differentiation. *J Neuroimmune Pharmacol* **7**, 750-762, doi:10.1007/s11481-012-9367-y (2012).

58 Yu, Q. *et al.* Role of beta-catenin in B cell development and function. *J Immunol* **181**, 3777-3783, doi:10.4049/jimmunol.181.6.3777 (2008).

59 Scheller, M. *et al.* Hematopoietic stem cell and multilineage defects generated by constitutive beta-catenin activation. *Nat Immunol* **7**, 1037-1047, doi:10.1038/ni1387 (2006).

60 Baba, Y. *et al.* Constitutively active beta-catenin promotes expansion of multipotent hematopoietic progenitors in culture. *J Immunol* **177**, 2294-2303, doi:10.4049/jimmunol.177.4.2294 (2006).

61 Luis, T. C. *et al.* Wnt3a deficiency irreversibly impairs hematopoietic stem cell self-renewal and leads to defects in progenitor cell differentiation. *Blood* **113**, 546-554, doi:10.1182/blood-2008-06-163774 (2009).

62 Swann, J. B., Happe, C. & Boehm, T. Elevated levels of Wnt signaling disrupt thymus morphogenesis and function. *Sci Rep* **7**, 785, doi:10.1038/s41598-017-00842-0 (2017).

63 Garcia-Perez, L. *et al.* Functional definition of a transcription factor hierarchy regulating T cell lineage commitment. *Sci Adv* **6**, eaaw7313, doi:10.1126/sciadv.aaw7313 (2020).

64 Rando, T. A. Stem cells, ageing and the quest for immortality. *Nature* **441**, 1080-1086, doi:10.1038/nature04958 (2006).

65 Nixon, A. B. *et al.* Peripheral immune-based biomarkers in cancer immunotherapy: can we realize their predictive potential? *J Immunother Cancer* **7**, 325, doi:10.1186/s40425-019-0799-2 (2019).

66 Pai, S. Y. *et al.* Transplantation outcomes for severe combined immunodeficiency, 2000-2009. *N Engl J Med* **371**, 434-446, doi:10.1056/NEJMoa1401177 (2014).

67 De Kouchkovsky, I. & Abdul-Hay, M. 'Acute myeloid leukemia: a comprehensive review and 2016 update'. *Blood Cancer J* **6**, e441, doi:10.1038/bcj.2016.50 (2016).

68 Morris, E. C. Allogeneic hematopoietic stem cell transplantation in adults with primary immunodeficiency. *Hematology Am Soc Hematol Educ Program* **2020**, 649-660, doi:10.1182/hematology.2020000152 (2020).

69 Hierlmeier, S., Eyrich, M., Wölfl, M., Schlegel, P. G. & Wiegering, V. Early and late complications following hematopoietic stem cell transplantation in pediatric patients - A retrospective analysis over 11 years. *PLoS One* **13**, e0204914, doi:10.1371/journal.pone.0204914 (2018).

70 Styczyński, J. *et al.* Death after hematopoietic stem cell transplantation: changes over calendar year time, infections and associated factors. *Bone Marrow Transplant* **55**, 126-136, doi:10.1038/s41409-019-0624-z (2020).

71 Yu, V. W. *et al.* Specific bone cells produce DLL4 to generate thymus-seeding progenitors from bone marrow. *J Exp Med* **212**, 759-774, doi:10.1084/jem.20141843 (2015).

72 Ito, R. *et al.* Late Effects of Exposure to Ionizing Radiation and Age on Human Thymus

Morphology and Function. *Radiat Res* **187**, 589-598, doi:10.1667/rr4554.1 (2017).

73 Wils, E. J. *et al.* Insufficient recovery of thymopoiesis predicts for opportunistic infections in allogeneic hematopoietic stem cell transplant recipients. *Haematologica* **96**, 1846-1854, doi:10.3324/haematol.2011.047696 (2011).

74 Moutuou, M. M., Pagé, G., Zaid, I., Lesage, S. & Guimond, M. Restoring T Cell Homeostasis After Allogeneic Stem Cell Transplantation; Principal Limitations and Future Challenges. *Front Immunol* **9**, 1237, doi:10.3389/fimmu.2018.01237 (2018).

75 Le Blanc, K. *et al.* Lymphocyte recovery is a major determinant of outcome after matched unrelated myeloablative transplantation for myelogenous malignancies. *Biol Blood Marrow Transplant* **15**, 1108-1115, doi:10.1016/j.bbmt.2009.05.015 (2009).

76 Maury, S. *et al.* Prolonged immune deficiency following allogeneic stem cell transplantation: risk factors and complications in adult patients. *Br J Haematol* **115**, 630-641, doi:10.1046/j.1365-2141.2001.03135.x (2001).

77 Velardi, E., Tsai, J. J. & van den Brink, M. R. M. T cell regeneration after immunological injury. *Nat Rev Immunol* **21**, 277-291, doi:10.1038/s41577-020-00457-z (2021).

78 Takahama, Y. Journey through the thymus: stromal guides for T-cell development and selection. *Nat Rev Immunol* **6**, 127-135, doi:10.1038/nri1781 (2006).

79 Beerman, I., Maloney, W. J., Weissmann, I. L. & Rossi, D. J. Stem cells and the aging hematopoietic system. *Curr Opin Immunol* **22**, 500-506, doi:10.1016/j.coi.2010.06.007 (2010).

80 Montecino-Rodriguez, E. *et al.* Lymphoid-Biased Hematopoietic Stem Cells Are Maintained with Age and Efficiently Generate Lymphoid Progeny. *Stem Cell Reports* **12**, 584-596, doi:10.1016/j.stemcr.2019.01.016 (2019).

81 Nikolich-Žugich, J. Author Correction: The twilight of immunity: emerging concepts in aging of the immune system. *Nat Immunol* **19**, 1146, doi:10.1038/s41590-018-0205-0 (2018).

82 den Braber, I. *et al.* Maintenance of peripheral naive T cells is sustained by thymus output in mice but not humans. *Immunity* **36**, 288-297, doi:10.1016/j.immuni.2012.02.006 (2012).

83 Mold, J. E. *et al.* Cell generation dynamics underlying naive T-cell homeostasis in adult humans. *PLoS Biol* **17**, e3000383, doi:10.1371/journal.pbio.3000383 (2019).

84 Flores, K. G., Li, J., Sempowski, G. D., Haynes, B. F. & Hale, L. P. Analysis of the human thymic perivascular space during aging. *J Clin Invest* **104**, 1031-1039, doi:10.1172/jci7558 (1999).

85 Ogonek, J. *et al.* Immune Reconstitution after Allogeneic Hematopoietic Stem Cell Transplantation. *Front Immunol* **7**, 507, doi:10.3389/fimmu.2016.00507 (2016).

86 Abdel-Azim, H., Elshoury, A., Mahadeo, K. M., Parkman, R. & Kapoor, N. Humoral Immune Reconstitution Kinetics after Allogeneic Hematopoietic Stem Cell Transplantation

in Children: A Maturation Block of IgM Memory B Cells May Lead to Impaired Antibody Immune Reconstitution. *Biol Blood Marrow Transplant* **23**, 1437-1446, doi:10.1016/j.bbmt.2017.05.005 (2017).

87 McManigle, W., Youssef, A. & Sarantopoulos, S. B cells in chronic graft-versus-host disease. *Hum Immunol* **80**, 393-399, doi:10.1016/j.humimm.2019.03.003 (2019).

88 Park, B. G. *et al.* Reconstitution of lymphocyte subpopulations after hematopoietic stem cell transplantation: comparison of hematologic malignancies and donor types in event-free patients. *Leuk Res* **39**, 1334-1341, doi:10.1016/j.leukres.2015.09.010 (2015).

89 Scarselli, A. *et al.* Longitudinal Evaluation of Immune Reconstitution and B-cell Function After Hematopoietic Cell Transplantation for Primary Immunodeficiency. *J Clin Immunol* **35**, 373-383, doi:10.1007/s10875-015-0154-4 (2015).

90 Pao, M. *et al.* Response to pneumococcal (PNCRM7) and haemophilus influenzae conjugate vaccines (HIB) in pediatric and adult recipients of an allogeneic hematopoietic cell transplantation (alloHCT). *Biol Blood Marrow Transplant* **14**, 1022-1030, doi:10.1016/j.bbmt.2008.06.012 (2008).

91 Scholz, J. L., Diaz, A., Riley, R. L., Cancro, M. P. & Frasca, D. A comparative review of aging and B cell function in mice and humans. *Curr Opin Immunol* **25**, 504-510, doi:10.1016/j.coi.2013.07.006 (2013).

92 Rodriguez-Zhurbenko, N., Quach, T. D., Hopkins, T. J., Rothstein, T. L. & Hernandez, A. M. Human B-1 Cells and B-1 Cell Antibodies Change With Advancing Age. *Front Immunol* **10**, 483, doi:10.3389/fimmu.2019.00483 (2019).

93 Sasaki, S. *et al.* Limited efficacy of inactivated influenza vaccine in elderly individuals is associated with decreased production of vaccine-specific antibodies. *J Clin Invest* **121**, 3109-3119, doi:10.1172/jci57834 (2011).

94 Frasca, D., Van der Put, E., Riley, R. L. & Blomberg, B. B. Reduced Ig class switch in aged mice correlates with decreased E47 and activation-induced cytidine deaminase. *J Immunol* **172**, 2155-2162, doi:10.4049/jimmunol.172.4.2155 (2004).

95 Nicoletti, C., Borghesi-Nicoletti, C., Yang, X. H., Schulze, D. H. & Cerny, J. Repertoire diversity of antibody response to bacterial antigens in aged mice. II. Phosphorylcholine-antibody in young and aged mice differ in both VH/VL gene repertoire and in specificity. *J Immunol* **147**, 2750-2755 (1991).

96 Murasko, D. M. *et al.* Role of humoral and cell-mediated immunity in protection from influenza disease after immunization of healthy elderly. *Exp Gerontol* **37**, 427-439, doi:10.1016/s0531-5565(01)00210-8 (2002).

97 Phelan R, A. M., Chen M. Current use and outcome of hematopoietic stem cell transplantation: CIBMTR US summary slides. (2020). <www.cibmtr.org>.

98 Verbinnen, B., Covens, K., Moens, L., Meyts, I. & Bossuyt, X. Human CD20+CD43+CD27+CD5-

B cells generate antibodies to capsular polysaccharides of Streptococcus pneumoniae. *J Allergy Clin Immunol* **130**, 272-275, doi:10.1016/j.jaci.2012.04.040 (2012).

99 Lam, J. H. & Baumgarth, N. The Multifaceted B Cell Response to Influenza Virus. *J Immunol* **202**, 351-359, doi:10.4049/jimmunol.1801208 (2019).

100 Ghosn, E. E. *et al.* Distinct B-cell lineage commitment distinguishes adult bone marrow hematopoietic stem cells. *Proc Natl Acad Sci U S A* **109**, 5394-5398, doi:10.1073/pnas.1121632109 (2012).

101 Yoshimoto, M. *et al.* Embryonic day 9 yolk sac and intra-embryonic hemogenic endothelium independently generate a B-1 and marginal zone progenitor lacking B-2 potential. *Proc Natl Acad Sci U S A* **108**, 1468-1473, doi:10.1073/pnas.1015841108 (2011).

102 Kobayashi, M. *et al.* Functional B-1 progenitor cells are present in the hematopoietic stem cell-deficient embryo and depend on Cbfβ for their development. *Proc Natl Acad Sci U S A* **111**, 12151-12156, doi:10.1073/pnas.1407370111 (2014).

103 Düber, S. *et al.* Induction of B-cell development in adult mice reveals the ability of bone marrow to produce B-1a cells. *Blood* **114**, 4960-4967, doi:10.1182/blood-2009-04-218156 (2009).

104 Esplin, B. L., Welner, R. S., Zhang, Q., Borghesi, L. A. & Kincade, P. W. A differentiation pathway for B1 cells in adult bone marrow. *Proc Natl Acad Sci U S A* **106**, 5773-5778, doi:10.1073/pnas.0811632106 (2009).

105 Holodick, N. E., Repetny, K., Zhong, X. & Rothstein, T. L. Adult BM generates CD5+ B1 cells containing abundant N-region additions. *Eur J Immunol* **39**, 2383-2394, doi:10.1002/eji.200838920 (2009).

106 Quách, T. D. *et al.* Human B-1 and B-2 B Cells Develop from Lin-CD34+CD38lo Stem Cells. *J Immunol* **197**, 3950-3958, doi:10.4049/jimmunol.1600630 (2016).

107 Ghosn, E. E. *et al.* Fetal Hematopoietic Stem Cell Transplantation Fails to Fully Regenerate the B-Lymphocyte Compartment. *Stem Cell Reports* **6**, 137-149, doi:10.1016/j.stemcr.2015.11.011 (2016).