Dissertation

# Context-specific Roles of Infections and Interferons in Hematopoiesis and Myeloid Neoplasms

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# Context-specific Roles of Infections and Interferons in Hematopoiesis and Myeloid Neoplasms

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This work is dedicated to my father, Jayarajan,

to honor his loving memories and his values on hard work.

# Summary

Hematopoietic stem cells (HSCs) that reside in the bone marrow are known to be of vital importance in generating diverse peripheral blood cell types, both under homeostasis and stress-hematopoiesis. Stressors such as infections and inflammatory cytokines such as IFNα can have direct effects on HSC biology. This exemplifies the importance of these factors in stress-hematopoiesis, and hematological neoplasms driven by mutant HSCs. To this end, two different aspects of these factors were studied in this dissertation – The mechanism of action of IFNα as a therapeutic agent in the treatment of myeloproliferative neoplasm (MPN), and co-housing as a murine model system to study the effect of infections on HSCs.

The mechanism of action of pegylated-IFNα (peg-IFNα) in reducing mutant allelic burden in JAK2-mutated MPNs was studied, in comparison to JAK inhibitor therapy. Using a BrdU pulse-chase assay in a mouse model of MPN, it was observed that JAK2-mutant HSCs have an inherently higher proliferation compared to wildtype-HSCs. However, a sub-fraction of mutant HSCs was found to persist in a dormant state in the bone marrow. These dormant mutant HSCs may be responsible for disease propagation and offer resistance to therapy with JAK inhibitors such as Fedratinib. In support of this, dormant JAK2-mutant HSCs were unaffected by Fedratinib treatment in mice, where hematologic parameters had been normalized. On the other hand, peg-IFNα was found to eliminate the dormant mutant HSCs in concomitance with reduced mutant-allele burden. These data hence provide a potential mechanism of action of peg-IFNα in treating MPNs.

Chronic infections in mice have been shown to lead to an increased proliferation of the hematopoietic stem and progenitor cell (HSPC) compartment and a subsequent loss of stem cell function. However, many of these infection-models involve challenging mice with a very high dose of purified pathogen administered via a non-physiologic route, thereby not recapitulating a natural infection process. To this end, a co-housing model was assessed, which allows natural transmission of infections from so-called “wildling” mice, to specific-pathogen-free laboratory mice, termed “co-housed” mice. The wildling mice have a microbiota and pathogen spectrum, similar to that of a wild mouse, which they aquire by neonatal vertical transmission. The co-housing model

was observed to have an efficient horizontal transmission of multiple infections from wildling to co-housed mice. Both acute and chronic phases of co-housing mediated infections were observed to elevate the peripheral blood cell count in the co-housed mice, but did not lead to expansion of HSPCs in the bone marrow. The chronic infections acquired through co-housing were found to reduce the functional potency of HSCs in the co-housed mice. This was in line with observations in previous infection models studied by other groups, albeit to a milder degree. Furthermore, the effect of vertical transmission was studied in the wildling mice. It was found that the HSCs from these mice did not have any reduction in function, contrary to what was observed in the co-housed mice. This indicates that neonatal exposure to pathogens preserves HSC function, suggesting a context-specific window of opportunity for infection-mediated functional attrition of HSCs.

Overall, the findings from this doctoral dissertation have contributed to the field of hematology from two unique perspectives. This work has provided a new paradigm to further explore novel strategies to eliminate therapy resistant HSCs in the setting of JAK2 mutated MPNs. It has also led to the development of a model to study differential response of HSCs to infections depending on the timing of exposure to pathogens. Together, these contributions can drive development of new therapeutic options for MPN patients, and also further our understanding of the roles of infections in hematopoiesis and hematologic malignancies.

# Zusammenfassung

Es ist bekannt, dass hämatopoetische Stammzellen (HSZ), die sich im Knochenmark befinden, von entscheidender Bedeutung für die Bildung verschiedener peripherer Blutzelltypen sind, und zwar sowohl bei Homöostase als auch bei Stress-Hämatopoese. Stressfaktoren wie Infektionen und entzündliche Zytokine wie IFNα können direkte Auswirkungen auf die Biologie von HSZ haben. Dies verdeutlicht die Bedeutung dieser Faktoren bei der Stress-Hämatopoese und bei hämatologischen Neoplasien die durch mutierte HSZ ausgelöst werden. Zu diesem Zweck wurden in dieser Dissertation zwei verschiedene Aspekte dieser Faktoren untersucht - der Wirkmechanismus von IFNα als therapeutisches Mittel bei der Behandlung von myeloproliferativen Neoplasien (MPN) und Co-Housing als murines Modellsystem zur Untersuchung der Auswirkungen von Infektionen auf HSZs.

Der Wirkmechanismus von pegyliertem IFNα (peg-IFNα) bei der Verringerung der Mutationslast in JAK2-mutierten MPNs wurde im Vergleich zur Therapie mit dem JAK-Inhibitor Fedratinib untersucht. Mit Hilfe eines BrdU-Puls-Chase-Assays in einem MPN-Mausmodell wurde festgestellt, dass JAK2-mutierte HSZ im Vergleich zu Wildtyp-HSZ eine höhere Proliferationsrate aufweisen. Es wurde jedoch auch festgestellt, dass ein Teil der mutierten HSZ in einem ruhenden Zustand verbleibt. Diese ruhenden mutierten HSZ könnten für die Krankheit verantwortlich sein und eine Resistenz gegen die Therapie mit JAK-Inhibitoren wie Fedratinib erklären. Dies wird dadurch bekräftigt, dass die Behandlung mit Fedratinib bei den Mäusen, bei denen sich die hämatologischen Parameter normalisiert hatten, keine Auswirkung auf die ruhenden JAK2-mutierten HSZ hatte. Im Gegensatz dazu wurde festgestellt, dass peg-IFNα die ruhenden mutierten HSZ eliminiert und zu einer reduzierten Mutationslast führt. Die erhobenen Daten liefern somit einen potenziellen Wirkmechanismus von peg-IFNα bei der Behandlung von MPN.

Chronische Infektionen bei Mäusen führen nachweislich zu einer verstärkten Proliferation der hämatopoetischen Stamm- und Vorläuferzellen (HSVZ) und zu einem anschließenden Verlust der Stammzellfunktion. Bei vielen der verwendeten Infektionsmodellen werden die Mäuse jedoch mit einer sehr hohen Dosis an aufgereinigtem Pathogen konfrontiert, welches meist über einen unphysiologischen

Weg verabreicht wird, wodurch ein natürlicher Infektionsprozess nicht rekonstruiert werden kann. Zu diesem Zweck wurde ein Co-Housing-Modell geprüft, das die natürliche Übertragung von Infektionen von so genannten " Wildling-Mäusen " auf spezifisch-pathogenfreie Labormäuse, sogenannte „Co-Housed-Mäuse“, ermöglicht. Die Wildling-Mäuse haben von Geburt an ein Mikrobiome und ein Erregerspektrum, das dem einer Wildmaus ähnelt und welches sie durch neonatale vertikale Übertragung akquirieren. In dem Co-Housing-Modell wurde eine effiziente horizontale Übertragung von zahlreichen Infektionen von Wildling- auf Co-Housed-Mäuse beobachtet. Sowohl in der akuten als auch in der chronischen Phase der durch Co-Housing vermittelten Infektionen wurde beobachtet, dass die Anzahl der peripheren Blutzellen in den Co-Housing-Mäusen anstieg, dies jedoch nicht zu einer Vermehrung der HSVZ im Knochenmark führte. Es wurde festgestellt, dass die durch Co-Housing erworbenen chronischen Infektionen die funktionelle Potenz der HSZ in den Co-Housing-Mäusen verringerte. Dies steht im Einklang mit Beobachtungen in früheren Infektionsmodellen, die von anderen Gruppen untersucht wurden, wenn auch in einem milderen Ausmaß. Darüber hinaus wurde auch der Effekt vertikaler Übertragung von Infektionen in den Wildling-Mäusen untersucht. Es wurde festgestellt, dass die HSZ dieser Wildling-Mäuse im Gegensatz zu den Co-Housing-Mäusen keine Funktionseinschränkung aufwiesen. Dies bedeutet, dass die neonatale Exposition gegenüber Krankheitserregern die Funktion von HSZ bewahrt, was auf ein kontextspezifisches Zeitfenster für die infektionsbedingte Funktionsreduktion der HSZ hindeutet.

Insgesamt haben die Ergebnisse dieser Dissertation in zweierlei Hinsicht einen Beitrag zum Fachgebiet der Hämatologie geleistet. Diese Arbeit hat ein neues Paradigma für die weitere Erforschung neuer Strategien zur Eliminierung therapieresistenter HSZ bei JAK2-mutierten MPN geliefert. Außerdem wurde ein Modell zur Untersuchung der Reaktion von HSZ auf Infektionen in Abhängigkeit vom Zeitpunkt der Exposition gegenüber Krankheitserregern entwickelt. Gemeinsam können diese Beiträge die Entwicklung neuer therapeutischer Optionen für MPN-Patienten vorantreiben und unser Verständnis der Rolle von Infektionen bei der Blutbildung und bei hämatologischen Malignomen erweitern.

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# 1 Introduction

## 1.1. Adult hematopoiesis

Blood is considered as one of the most regenerative tissues in mammals. This regenerative potential is required for the continued production of the different blood cells throughout the lifetime of an organism. Over $10^5$ erythrocytes and $10^4$ white blood cells are produced per second in an adult human under steady-state conditions (1). The blood is composed of cells arising from 4 main lineages, namely, myeloid lineage, lymphocytic lineage, erythroid lineage and megakaryocytic lineage (2). The cell types within these lineages have distinct functions in an adult. For example, the myeloid and lymphocytic lineages are responsible for innate and adaptive immune responses (3), the erythrocytes carry oxygen to tissues and organs (4), and the platelets produced from megakaryocytes are involved in blood coagulation and blood vessel homeostasis during injuries (5). The production of these diverse cell types is regulated through a hierarchical differentiation cascade of more primitive or precursor cells, as represented by the so-called hematopoietic hierarchy.

### 1.1.1. Hematopoietic hierarchy

The classical model of the hematopoietic hierarchy defines a tree-like differentiation cascade with well-defined differentiation branch points, starting from the long-term repopulating hematopoietic stem cells (LT-HSCs) at the top, to the final terminally differentiated cell types of the peripheral blood **(Figure 1.1)**. According to this model in mice, the LT-HSCs further give rise to the short-term repopulating HSCs (ST-HSCs) which in turn give rise to a range of multipotent progenitor cells (MPPs) (6). Over the years, the MPPs have been further subdivided into a range of MPPs such as MPP2, MPP3, MPP4 and MPP5, based on the expression of specific cell-surface markers (7). These cell types from the LT-HSCs to the MPPs are referred to as the

hematopoietic stem and progenitor cells (HSPCs). This differentiation from LT-HSCs to MPPs is characterized by a very sharp decline in the self-renewal capacity (8). The first bifurcation in the lineage commitment has been defined from the MPPs giving rise to the common myeloid progenitors (CMPs) and the common lymphoid progenitors (CLPs) (9,10). The CMPs are characterized by their ability to only differentiate into myeloid, erythroid and megakaryocytic lineages, while the CLPs are characterized by their ability to differentiate into the lymphoid lineage. Further branching is proposed in differentiation potential from the CMP into the megakaryocyte-erythrocyte progenitors (MEPs) and the granulocyte-monocyte progenitors (GMPs) (11,12). Together, the CMPs, CLPs, GMPs and MEPs are referred to as the oligo-potent progenitors. Along this cellular compartment there is a marked decrease in multi-lineage differentiation potential accompanied by commitment to differentiation to a specific hematopoietic lineage. These progenitors further give rise to more unipotent precursors called lineage-restricted progenitors that finally differentiate into one of the diverse mature blood cell types such as granulocytes, monocytes, erythrocytes, platelets, T-lymphocytes, B-lymphocytes, etc. (13)

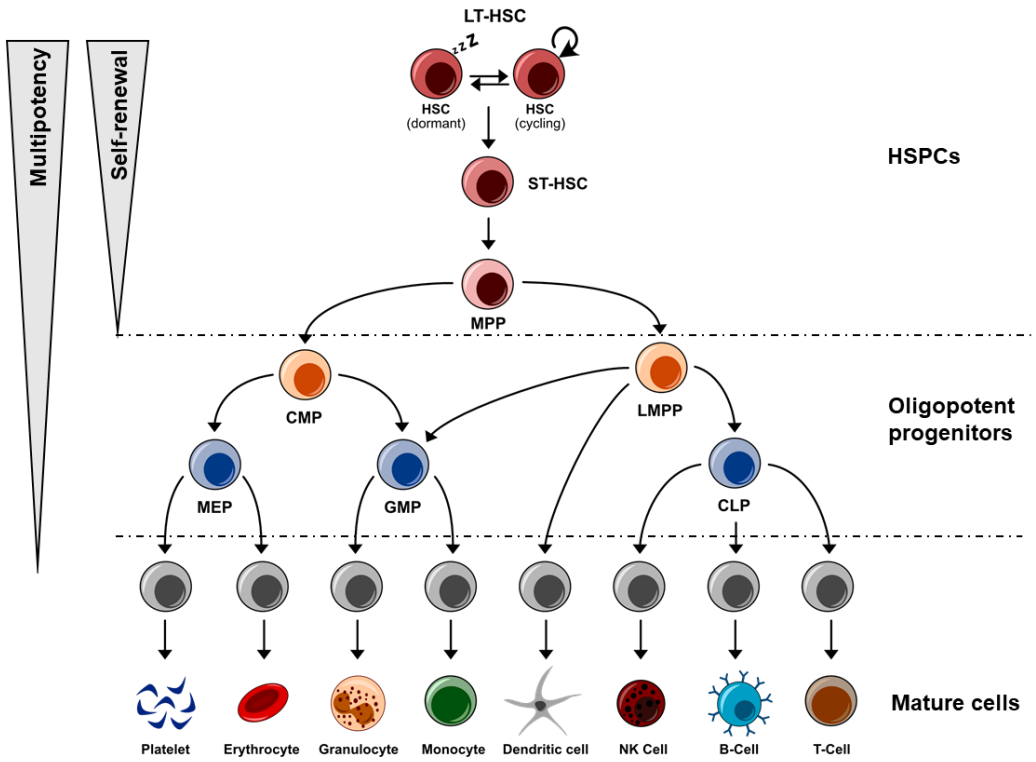


Figure 1.1: Classical model of the hematopoietic hierarchy

The defined branch points and bifurcations in the differentiation cascade specified by the classical model of the hematopoietic hierarchy has been highly contested over recent years with the advent of single cell RNA-sequencing technologies and single-cell HSC transplantation experiments (14–18). These approaches have been used to propose the so-called continuum model of hematopoiesis. According to this model, the HSCs still sit at the apex but subsequent differentiation occurs through a rather continuous flux of heterogeneous cellular states that gradually commit to a specific lineage, as opposed to the abruptly defined branch points. Also the immunophenotypically defined HSCs have been shown to be a mix of heterogeneous cells, already possessing a certain degree of bias towards differentiating into a specific cellular lineage. Studies have shown the existence of HSCs that are specifically primed towards lymphoid, myeloid and megakaryocytic lineages even under steady-state conditions (19–23). Nevertheless, transcriptional states cannot be used to effectively identify and isolate lineage-biased HSCs or the continuum of differentiating cells through flow cytometry, due to lack of specific cell surface markers. Hence, for the purpose of overall identification of cells along differentiation trajectories of differentiation, the hierarchical order defined by the classical model is still under use.

### 1.1.2. Hematopoietic Stem Cells

Hematopoietic stem cells (HSCs) sitting at the top of the hematopoietic hierarchy are conventionally defined based on two central characteristics. Firstly, they possess the capacity of "multipotency" to differentiate and repopulate all the different hematopoietic lineages. Secondly, they have the distinctive ability of "self-renewal", through which they are able to generate more HSCs (13). These distinct characteristics of multipotency and self-renewal reduces as the HSCs start differentiating into further cell types down the hematopoietic hierarchy. Also, the ability of self-renewal is known to drop drastically as the HSCs differentiate into MPPs (13). Immunophenotypically, murine HSCs are identified as Lineage- Sca1+ cKit+ CD150+ CD48- cells and are further subdivided as ST-HSC and LT-HSC based on their higher or lower CD34 expression respectively (24). The LT-HSCs in the mouse bone marrow differ from the ST-HSCs in that they are capable of long-term repopulation of bone marrow when transplanted into recipient mice. ST-HSCs on the other hand lead to graft failure over time due to their limited self-renewal ability (24).

LT-HSCs in murine bone marrow have been shown to primarily exist in a dormant state and rarely divide under homeostatic conditions (25). In fact, steady-state hematopoiesis has been shown through label-tracing experiments to be predominantly driven by downstream MPP populations (26). The dormancy of LT-HSCs under unperturbed hematopoiesis is considered to be a protective mechanism to safeguard their functional potency to repopulate the blood lineages when required to do so. For example, certain conditions of stress on the hematopoietic system has been shown to push dormant HSCs into cycle to facilitate the increased need for blood production (27–30). This push out of dormancy however, comes at the price of reduction in HSC functional fitness due to replication-stress induced DNA damage (28). Dormant HSCs transplanted into lethally irradiated mice have been shown to possess better bone marrow engraftment and functional potency, compared to HSCs that have proliferated (25). This places emphasis on the role of perturbations in hematopoiesis and their deleterious effects on HSC function.

## 1.2. Stress-hematopoiesis and its effect on HSCs

Different conditions of stress such as inflammation, infections, blood loss, chemotherapy, etc., lead to an increased rate of bone marrow hematopoiesis, in what is called stress-hematopoiesis, to meet the high demand for peripheral blood cell production. For example, systemic infections mediate an increased myeloid cell output, in what is referred to as “emergency granulopoiesis”. This involves the massive proliferation and differentiation of committed myeloid progenitors and the subsequent engagement of the hematopoietic stem and progenitor cell (HSPC) compartment (31). The proliferation of HSPCs is required since myeloid cells are post-mitotic and can only be generated through differentiation of progenitor cells (32). Furthermore, injecting mice with pI:pC, a dsRNA-memetic that elicits an inflammatory response, has been shown to directly drive HSCs into megakaryocyte differentiation (33). This indicates the direct involvement of the HSCs that sit at the apex of the hematopoietic hierarchy, in combating conditions of stress. Stress-hematopoiesis over a short period of time such as inflammatory response to acute infection does not lead to a reduction in HSC functional potency (34). This highlights the great tolerance of the hematopoietic system, in particular the HSCs, in responding to acute stress and is vital to the survival of an organism. However, as previously mentioned, HSCs in the

bone marrow normally exist in a dormant state under homeostasis, but are driven into active proliferation during chronic phases of stress-hematopoiesis **(Figure 1.2)**. This increased proliferation of HSCs has been shown in mice to significantly decrease its functional potency, when the stressor is sustained over long periods of time. With the known adverse effects on HSC fitness related to these stressors, it makes it vital to understand the mechanisms as to how different stressors alter hematopoiesis and affect HSC biology.

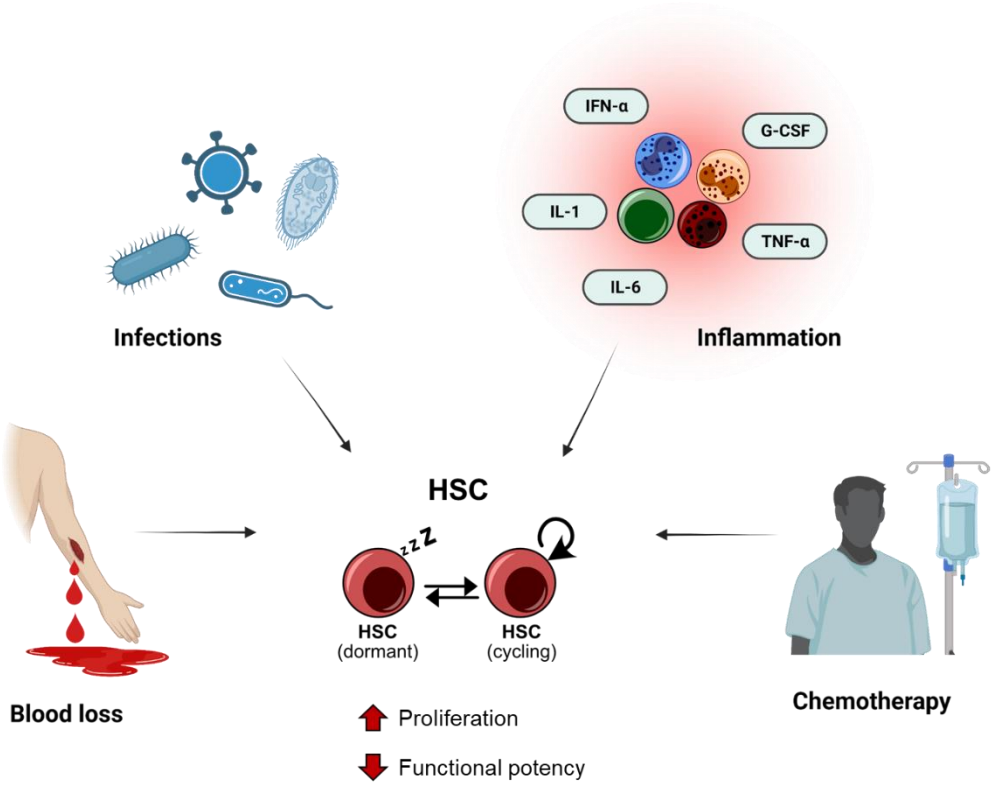


**Figure 1.2: Different stressors of hematopoiesis can push dormant HSCs into proliferation and reduce their functional potency over time**
*(Image partially created using Biorender.com)*

The effects of stress-hematopoiesis are not limited to the bone marrow. The increased proliferation of HSPCs in the bone marrow during stress-hematopoiesis is also accompanied by the increased egress of HSPCs from the bone marrow. Stressors such as infections, pregnancy, anemia and even metabolic stress has been shown to induce mobilization of HSPCs into peripheral blood and distal organs (35–37). This mobilization of HSPCs results in active hematopoiesis in these extramedullary organs, as an alternative or in addition to bone marrow hematopoiesis, and is referred to as extramedullary hematopoiesis (EMH). The most common organs of extramedullary

hematopoiesis in adults have been noted to be the spleen, liver and the lymph nodes. These organs are known sites of hematopoiesis during embryonic and post-natal hematopoiesis (38,39), indicating a potential reason as to why EMH happens in these organs. Even though splenic EMH is negligible under steady-state, plethora of conditions of stress such as anemia, infection, myeloablation, atherosclerosis, colitis, diabetes, cardiovascular diseases, etc., have all been associated with EMH at the spleen (29,40–45). Splenic EMH and the mechanisms of HSPC proliferation, and subsequent hematopoiesis in the spleen, has hence been widely studied over the years (46). EMH acts as a compensatory mechanism to meet the failure of the bone marrow to achieve the required hematopoietic demand (47,48). However, chronic demand on EMH can be detrimental to these organs and their normal function. EMH is also frequently observed in hematological malignancies such as myeloproliferative neoplasms. The precise mechanisms underlying EMH and ways of mitigating EMH during stress is hence an area that still requires further attention.

### 1.2.1. Inflammatory cytokines as mediators of stress-hematopoiesis

The role of inflammation and inflammatory cytokines in hematopoiesis has always been a topic of great interest to many researchers. Multiple perspectives of inflammatory cytokines, from embryonic hematopoiesis to adult steady-state hematopoiesis to stress-hematopoiesis, has been widely studied over the years. Some inflammatory cytokines commonly associated to elicit effects on HSCs include interferons (IFN), TNFα, interleukins and G-CSF (49).

Interferons such as IFNα, IFNß and IFNγ are cytokines that were originally thought to only function in anti-viral activity during an immune response. However, many other functional roles in hematopoiesis have now been attributed to IFNs. For example, IFNα has been found to be important for the emergence and maturation of HSCs in developing embryos (50). The first evidence for the role of IFNs in adult hematopoiesis was observed in lymphocytic choriomeningitis virus (LCMV), where progenitor cell depletion following infection could be prevented upon IFN receptor knockout (51). Further studies on treating mice directly with IFNα has shown that it pushed dormant HSCs into proliferation, with chronic treatment leading to attrition of HSC function (27). Furthermore, it has also been shown that IRF2, a factor that represses type I IFN receptors, are required for maintaining HSC quiescence (52). IFNγ has also been observed to play a role in *Mycobacterium avium* and *Ehrlichia*

*muris* infection mediated HSPC proliferation and expansion in mice (30,53). These findings among others have established interferons as primary players in regulating HSC dormancy and function under both steady-state and infection-induced hematopoiesis.

Tumor necrosis factor-alpha (TNFα) is another pro-inflammatory cytokine that are produced by immune cells and endothelial cells. It has a multitude of functions ranging from regulation of apoptosis, to pyrogenic functions (54,55). *In vivo* experiments in mice have shown that TNFα acts as another important regulator of adult hematopoiesis. Studies have shown that treating mice with TNFα can lead to reduction in HSC proliferation and ability to repopulate the hematopoietic system (12). However, the role of TNFα in hematopoiesis is still controversial with some studies claiming that TNFα improves HSC engraftment and function in transplants (56). With TNFα having two different extracellular receptors expressed, it might have differential effects on HSCs in a context-dependent manner.

Pro-inflammatory interleukins such as IL-1β and IL-3 have also been widely implicated to be important in adult bone marrow hematopoiesis. Chronic exposure of mice to IL-1 has been shown to induce HSC proliferation and differentiation into myeloid lineage (57). This also led to a decline in HSC repopulation ability. However, this effect was observed to be transient. Stopping IL-1 administration was found to replenish HSC self-renewal and functional potency. Similar findings of HSC expansion has also been reported in mice with IL-3 and IL-27 (58,59), suggesting the role of these common inflammatory mediators in HSC proliferation and function.

Granulocyte colony stimulating factor (G-CSF) is a glycoprotein that is known to promote granulopoiesis in response to infections (60). In addition to inducing granulopoiesis, G-CSF has long been known to induce mobilization of HSPCs, and hence is used as a way of enriching HSPCs in blood for stem cell transplantation in humans (61). Furthermore, G-CSF treatment in mice has been shown to induce HSC expansion and loss of functional potency, similar to other cytokines discussed above (62). This effect was also shown to be mediated through toll-like-receptors (TLRs).

Together, these observations have all shown that all these cytokines have a common role in directly or indirectly inducing HSC proliferation. The presence of these pro-inflammatory cytokines at the apex of multitude of infection responses, underpins their significance in regulating HSC biology upon acute and chronic infection-stress.

### 1.2.2. Infection-induced hematopoiesis and its effects on HSCs

An individual experiences multiple infections, and the accompanied immune response, over the course of the person's lifetime. This makes it one of the most common stressors of the hematopoietic system due to the high demand for production of the different peripheral blood mature cells. The known deleterious effects of stress-hematopoiesis on the bone marrow and the HSCs, have hence warranted a plethora of studies to understand the effects of the process of infection response on the HSCs in particular.

Infections have been observed to have a profound impact on the bone marrow in general. For example, a typical bone marrow response that occurs in response to different types of infections is increased granulopoiesis to generate more neutrophils, basophils and eosinophils, in what is referred to as emergency granulopoiesis (63–65). There is also an increased myelopoiesis in the bone marrow leading to elevated production of monocytes and macrophages. The macrophages in the bone marrow have been observed to have an important role in maintaining the HSC niche, in addition to direct antimicrobial activities (66,67). For example, studies of *Toxoplasma gondii* infections in mice has proven the necessity of monocyte mobilization from the bone marrow to clear these infections, and studies on *Ehrlichia* infections have shown the dependence on IFNγ-induced macrophage activation in clearing infections (53,68). Furthermore, suppression of erythropoiesis leading to anemia has been observed in certain infections such as *Ehrlichia muris* and the African trypanosomes (69,70). In addition to these myeloid cells, infections can also lead to changes in the bone marrow lymphocytes. Infections are known to significantly impact on B-cell development in the bone marrow. As an example, *Trypanosoma brucei* infections in mice has been shown to be responsible for reduced B-lymphopoiesis in the bone marrow (71). Infection of mice with influenza or LCMV virus has also shown to reduce the number of immature B-cells in the bone marrow (72,73), further indicating the direct effects of infection on bone marrow lymphopoiesis. In addition to B-cells, memory T-cells are also known to be present in the bone marrow, and have been shown to be highly activated and poly-functional compared to those present in peripheral blood (74,75). All these observations have validated the response of different bone marrow mature cells to infections. However, which specific mature cell populations are involved in infection

response are dependent to an extent, on the type of pathogen driving the infection response.

Among all the different infection models used to study infections in mice, one common effect that has been observed is the effect on the expansion of the HSPC compartment in the bone marrow. Many pathogenic infections in mice have been found to induce proliferation of dormant HSCs and can lead to decline in HSC functional potency upon chronic infections. Some of these studied infection models include injecting mice with pI:pC (viral dsRNA mimetic) and lipopolysaccharide (bacterial membrane component), directly injecting mice with highly pathogenic microorganisms such as *Mycobacterium avium* and murine cytomegalovirus (mCMV) or using mosquitoes as vectors to infect mice with *Plasmodium berghei* (28,30,76–78). Infections lead to the upregulation of a plethora systemic cytokines, all of which have direct effects on HSC proliferation and function, as previously discussed. Moreover, HSCs have also been shown to directly respond to infections. The surface of HSCs are known to possess the toll-like-receptors (TLRs) that detect pathogen associated molecular patterns (PAMPs) (79). Furthermore, the presence of TLR4 on HSCs have been observed to be essential for infection-induced HSC proliferation in mice injected with LPS or with *Pseudomonas aeruginosa* (80). These findings highlight the direct sensing of infections by HSCs and also the importance of understanding how infections lead to HSC exhaustion.

However, not all humans who undergo infections have dramatic ill-effects on HSCs leading to conditions such as bone marrow failure. This casts some doubt on the validity of extent of dramatic effects seen on HSCs with the existing infection models. The major caveat with all of the current mouse models to assess infections is that they either use a compound that only mimics an infection (pI:pC and LPS) or use injections of non-physiologically high-dose of purified pathogens into mice. This does not recapitulate a natural process of infection initiation or response that normally occurs in humans who acquire less severe and common infections on a day to day basis. With the increased incidences of hematological disorders with age, there appears to be a cumulative effect of multiple types of infections and inflammatory responses on HSCs over a long period of time. This shifts focus into assessing more natural milder transmission of microbes in mice, which would more accurately model infection-stress induced hematopoiesis.

## 1.3. Co-housing model of infection transmission

For multiple decades now, laboratory mice used for research are housed under very clean specific-pathogen-free (SPF) conditions as a way to standardize experimental conditions (81,82). However, this makes extrapolation of findings to human biology since humans rarely exist in such clean environments and are usually exposed to a variety of commensals and pathogens right from birth. Furthermore, many studies over recent years have emphasized the importance of the microbiome of an individual in the pathogenesis of a multitude of human diseases (83). This called for further attention to the use of SPF mice and the interpretation of scientific data generated using these mice, and its translation to human disease pathogenesis. Beura *et al.* were able to show that the peripheral immune landscape of SPF laboratory mice resemble that of a new born human child rather than that of an adult human (84). Moreover, wild mice are known to have a much more activated immune system with higher levels of serum antibody levels compared to laboratory mice housed in pathogen-free environments (85,86). These observations led to a few studies looking at ways to normalize the microbial load carried by laboratory mice. One such approaches included artificial infection of laboratory mice with herpes virus, influenza virus and helminth in a sequential manner. After this sequence of infections, the mice were observed to have the peripheral immune system that was completely reshaped to resemble that of adult humans (87). Even though this system allows precise control of which microbes and parasites are resident in the mice, this still goes against the natural transmission of multiple infections as it occurs in nature with humans. The approach of fecal transplantation to alter the gut microbiome has also been attempted (88). Laboratory mice in this study was administered with fecal slurry from wild mice through oral gavage. Nevertheless, the gut microbiome is only a fraction of the rich microbiome spectrum of an organism.

Recently, the approach of co-housing to facilitate microbiome and pathogen transfer was trialed by Beura *et al* (84). In their study, the researchers housed a mouse acquired from a pet-store within the same cage as a laboratory mouse. The pet-store mice having been housed and raised under non-pathogen-free environments, were observed to have a variety of commensals and pathogens in them. These mice hence have a more activated peripheral immune system, with increased proportions of active and memory T-cells for example. Co-housing the pet-store mice with the laboratory mice was shown to allow efficient transfer of many of the infections from the pet-store

mice onto the laboratory mice. Furthermore, this led to increased peripheral immune activation of the laboratory mice, and the proportions of different T-cell subsets was found to resemble that of an adult human. All the work done by them only involved assessing the change and normalization of the peripheral immune cells and did not assess any changes in the bone marrow in the co-housed mice. They also performed transcriptomics analysis on the peripheral blood mononuclear cells (PBMCs) of these co-housed mice and adult human PBMCs. Their gene enrichment analysis comparing these two experimental groups showed significant enrichment of gene signatures associated to type-I interferon signaling, interferon production, IFNα response, upregulation of cytokine production, etc. (84) All of these responses are not just known to affect the peripheral immunity, but also directly influence hematopoietic stem and progenitor cells in the bone marrow, as described extensively in previous sections. This led to the hypothesis in this thesis, that the co-housing model would be suitable for assessing the effects of natural transmission of infections on hematopoiesis and HSC biology in mice. The model here would represent the horizontal transmission of infections to adult laboratory mice that are immunologically naïve before co-housing.

## 1.4. Wildling-mice that carry wild-mouse microbiota

The use of pet-store mice as a model of wild-microbiota for co-housing has a caveat, that the pathogen spectrum is highly variable across different pet-store mice. Moreover, 22% of laboratory mice were observed to die after co-housing due to some highly virulent pathogens occasionally being transmitted (84). In 2019, Rosshart et al. found an elegant method to generate mice with wild-microbiota, while still maintaining the genetic tractability of a laboratory mouse (89). They implanted embryos from a C57BL/6 laboratory mouse strain into pseudo-pregnant wild mouse, and allowed the wild mouse to give birth to the laboratory mouse strain **(Figure 1.3)**. The resultant progeny mice are referred to as the "wildling" mice. The wildlings were found to have the entire range of pathogen and microbiome spectrum similar to that of wild mice. This included not just the gut microbiome, but also the vaginal, skin and oral microbiota. Furthermore, the wildling mice also had a range of commensal and pathogenic infections such as *Helicobacter pylori* infections in them in addition to parasites such as mites (89). The presence of wild microbiota was observed to affect the immune cell composition of multiple organs in the wildling mice.

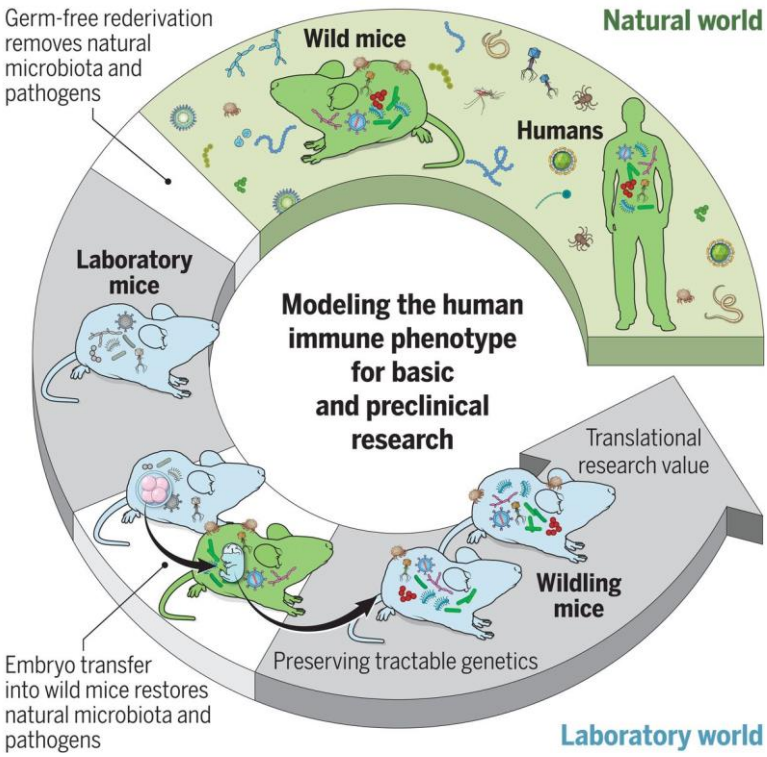


**Figure 1.3: Generation of wildling mice by implanting laboratory mouse embryo into wild-mice**
*(Image from (89); Reprinted with permission from AAAS)*

The wildling mice are now able to sustain the pathogen and microbiota spectrum just by breeding them together, thereby eliminating the need for further embryo re-derivation used to initially generate these mice. This made the wildling mice a suitable alternative to pet-store mice for co-housing based experiments. Since the wildling mice have the genetic background of the C57BL/6 laboratory mouse strain, it also makes them less prone to acclimatization issues with the laboratory mice during co-housing. The wildling mice provided by the group of Dr. Rosshart was hence used for co-housing experiments as part of this dissertation. The pet-store mice had the additional drawback that they were not genetically defined. This makes the direct analysis and interpretation of the pet-store mouse hematopoietic system difficult. However, the defined C57BL/6 background of the wildling mice gave me the opportunity to study an additional arm of infection transmission, namely vertical transmission. The wildling mice having had the infections vertically transmitted from mother, represent neonatal exposure to wild-microbiota infections. The differences in

hematopoiesis due to neonatal exposure to pathogens versus that as an adult is a research topic that has not been explored so far. The co-housing model and access to the wildling mice directly, hence would enable assessment of any differential effects of horizontal versus vertical transmission of infections on hematopoiesis and HSCs.

## 1.5. Clonal Hematopoiesis

Aging is accompanied by a gradual accumulation of somatic mutations in cells of different tissues, due to intrinsic reasons such as proliferation-induced DNA replication errors, and extrinsic factors such as environmental cues (90–92). These random somatic mutations that include base substitutions and indels, also occur in the hematopoietic stem cells over time (93–95). Most of the somatic mutations do not have any consequences on the HSCs. However, when a certain mutation offers a self-renewal or proliferative advantage over normal HSCs in the bone marrow, this can result in a clonal expansion without any apparent signs of a hematological disorder. This clonal expansion of a specific subset of HSCs is referred to as clonal hematopoiesis of indeterminate potential (CHIP). This phenomenon was first indicated by an observation that 25% of healthy women above 65 years of age show a skewed X-chromosome inactivation pattern in peripheral blood leukocytes (96). Individuals with CHIP have been observed to be at a high risk for developing cardiovascular diseases such as atherosclerosis, ischemic stroke, etc. (97). CHIP mutations have been in general correlated to an overall increase in mortality rate in individuals and offer poor prognosis in patients of different solid cancers (98,99). Furthermore, CHIP has been shown to be a significant risk factor to developing hematological disorders such as myelodysplastic syndrome (MDS), myeloproliferative neoplasms (MPN) and acute myeloid leukemia (AML) (100,101).

The most frequent mutations in individuals that exhibit clonal hematopoiesis have been observed to be mutations in epigenetic regulators such as *DNMT3A*, *TET2* and *ASXL1, and JAK2* mutations (102). The advent of sensitive next-generation sequencing technologies enabled identification of these CHIP mutations in hematological neoplasms, suggesting their role as pre-leukemic driver mutations that pave way for acquiring subsequent mutations that drive leukemogenesis. The mutation patterns suggest a clonal evolution that predisposes to but precedes leukemic transformation **(Figure 1.4)**. These pre-leukemic stem cells harboring possible

founder mutations can still undergo multi-lineage differentiation, often survive chemotherapy and may eventually expand to cause relapse (103).

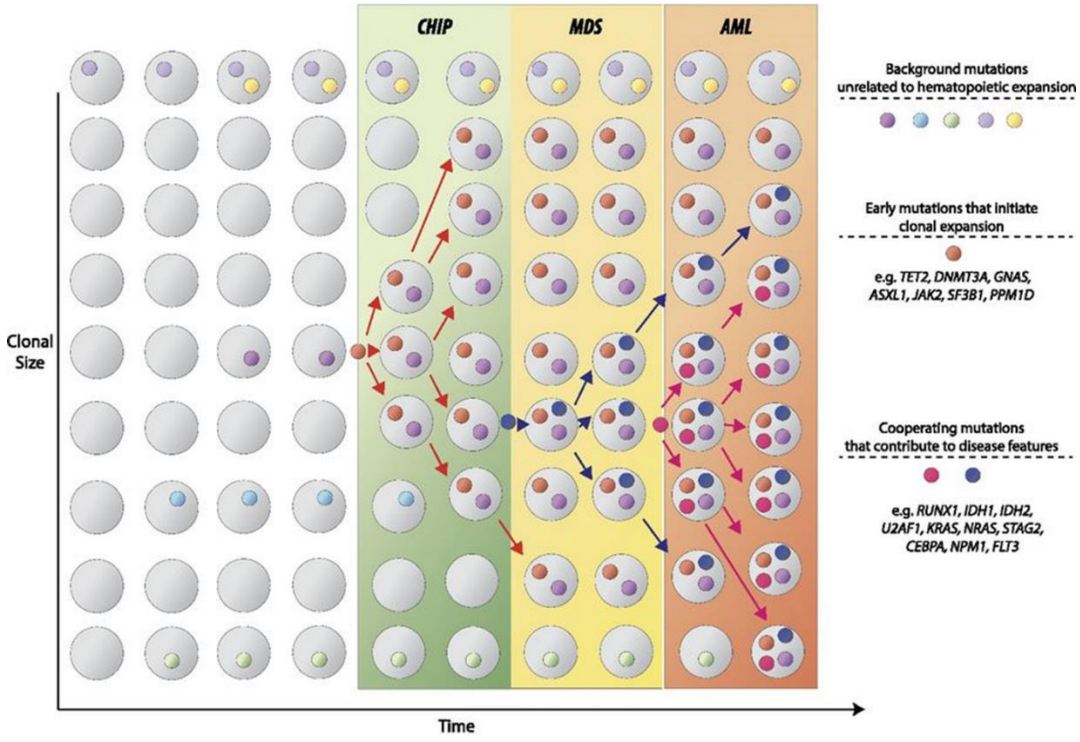


**Figure 1.4: Clonal hematopoiesis of indeterminate potential (CHIP) as a precursor to myeloid dysplasia and neoplasms**
*(Image from (104); Reprinted with permission from Elsevier through copyright.com)*

### 1.5.1. Factors influencing clonal expansion in CHIP

Many cell intrinsic and extrinsic factors have been shown to affect acquisition of CHIP mutations and drive the subsequent expansion of the mutant HSC clones. Like many hematological disorders, aging is a major driver of clonal hematopoiesis, predominantly due to the increase in accumulation of somatic mutations as an individual ages. Studies have shown that the number of CHIP-associated driver mutations increase exponentially with the age of an individual (100,101). Lifestyle factors such as smoking and obesity has also been shown to increase incidences of CHIP (105). In particular *ASXL1* mutated CHIP has been linked to smoking and TET2-assoicated CHIP has been linked to obesity-mediated insulin resistance (106,107). A major factor studied extensively of late is the effect of inflammation on

CHIP, and *vice versa*. Chronic inflammatory conditions such as chronic pulmonary disease, systemic sclerosis, rheumatoid arthritis and ulcerative colitis have all been linked to increased prevalence of clonal hematopoiesis (108–111). This has been linked to the elevated production of inflammatory cytokines such as TNFα and interferon-gamma (111). In addition to CHIP increasing the risk for cardiovascular diseases, cardiovascular diseases can also accelerate clonal hematopoiesis. It has been shown that the inflammatory state resulting from atherosclerosis can leads to acceleration of TET2-mediated clonal hematopoiesis in mouse models (112). Chronic infections are another factor that has been shown to exacerbate clonal hematopoiesis through inflammatory cytokines. Chronic *Mycobacterium* infections of *Dnmt3a*-mutated mouse model has shown to cause expansion of mutant HSCs in an interferon-gamma dependent manner (113). These observations emphasize the importance of inflammation as a major contributor to clonal hematopoiesis. Inflammatory cytokines and infections being two major factors that are well known to influence HSC proliferation and functional attrition, suggests that increased proliferation could force a selection pressure on HSC clones that have a proliferative advantage, leading to CHIP pathogenesis. Thus, understanding this process could be vital to further our understanding of CHIP and potential ways to curb the associated morbidities.

### 1.5.2. DNMT3A mutations in clonal hematopoiesis

DNA methyltransferase 3a (DNMT3A) is an important epigenetic modulator that catalyzes the methylation of cytosine residues in DNA. Mutations in *DNMT3A* are the most prevalent mutations found in individuals exhibiting clonal hematopoiesis. *DNMT3A* mutations can lead to increased HSC self-renewal and differentiation block, implicating its role as a CHIP-mutation (114). Mutations in *DNMT3A* have been shown to lead to increased inflammatory response to infections and the subsequent production of inflammatory cytokines such as IL-6 and IFN-γ (113). Furthermore, DNMT3A-mediated CHIP in humans has been shown to strongly influence cardiovascular disorder by production of pro-inflammatory cytokines, activation of inflammasomes and altering immune cell functions (115). Among the *DNMT3A* mutations, a specific mutational hotspot at R882 in the methyltransferase domain has been reported in 60% of cases. These mutations are heterozygous missense mutations, most commonly arginine to histidine substitutions (R882H) (116). The homologous R878H mutation in murine Dnmt3a has been shown to inhibit the methylation activity

of wild-type (WT) Dnmt3a in murine embryonic cells in a dominant-negative manner (117). Compared to the wildtype enzyme, Dnmt3a-R878H mutant shows only ~20% methyltransferase activity, potentially contributing to the phenotypes observed in mutant HSCs and downstream immune modulators.

### 1.5.3. TET2 mutations in clonal hematopoiesis

Ten-eleven translocation-2 (TET2) is an enzyme that plays a critical role in the demethylation of DNA CpG sites by catalyzing the oxidation of the methyl group. Mutations in TET2 are the second most common in CHIP. Similar to *Dnmt3a* mutations, loss of Tet2 in mice has been shown to increase HSC self-renewal and a myeloid bias, implicating its role in clonal hematopoiesis (118). Deficiency of TET2 has also been observed to induce a pro-inflammatory phenotype by increasing the levels of systemic inflammatory cytokines such as IL-1ß and IL-6 (107,119). Murine models have further shown that Tet2 loss leads to increased incidence of heart failure in mice through the NLRP3 inflammasome activation (120). The opposite effect of atherosclerosis-mediated inflammation accelerating Tet2-mediated CHIP phenotype has also been demonstrated in mice (107). These observations in TET2- and DNMT3A-mutant CHIP, implicates the intricate relationship between inflammatory cytokines and clonal hematopoiesis during aging. There appears to be a feedback network, wherein inflammation drives clonal hematopoiesis and clonal hematopoiesis drives inflammation.

### 1.5.4. JAK2 mutations in clonal hematopoiesis

Unlike *DNMT3A* and *TET2* mutations, that are more frequently observed in older individuals, mutations in the Janus kinase 2 (JAK2) are found in younger individuals. Individuals with JAK2-mediated CHIP have a 10-fold higher risk of cardiovascular disease and is one of the highest ranking risk factor for premature cardiac problems (97,121). *JAK2* mutations are also known to increase risk of thrombosis through the mechanism of neutrophil-extracellular-traps (122). In line with other CHIP mutations, *JAK2* mutations are also associated with increased levels of pro-inflammatory cytokines such as IL-6 and IL-18, reaffirming the role of inflammation in clonal hematopoiesis (121). However, in contrast to other common CHIP mutations, *JAK2* mutations are known to drive myeloid neoplasms without any

additional mutations. *JAK2* mutations are among the most frequently observed mutations in myeloproliferative neoplasm (123), suggesting the intriguing dual role of the mutation in CHIP and subsequent neoplasms.

## 1.6. Myeloproliferative neoplasms

Myeloproliferative neoplasms (MPNs) are a type of chronic leukemia, and are broadly categorized as Philadelphia-chromosome (Bcr-Abl gene translocation) positive and negative MPNs. Philadelphia-chromosome positive MPNs are referred to as Chronic Myeloid Leukemia (CML). The Philadelphia chromosome negative MPNs are the classical MPNs, and are further sub-divided into polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF). This categorization is based on the phenotypic differences observed in patients, with PV leading to increased red blood cell counts, ET leading to increased platelet counts, and PMF leading to bone marrow fibrosis and failure. (124)

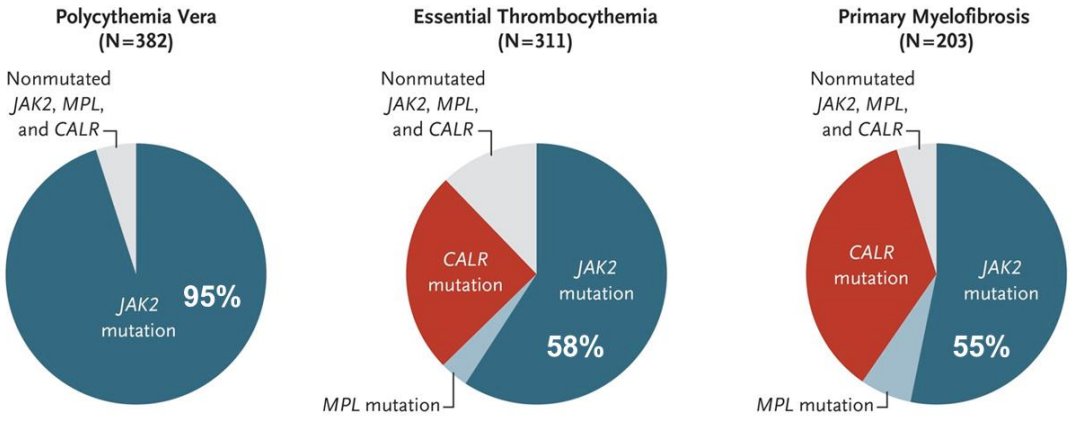


**Figure 1.5: Distribution of mutations in patients with polycythemia vera, essential thrombocythemia and myelofibrosis**
*(Image reprinted with permission from* (125)*; Copyright Massachusetts Medical Society)*

PMF is the most debilitating of MPNs, and both PV and ET patients have a high risk of progression towards secondary myelofibrosis. Furthermore, patients with all three types of malignancies also have a high risk of developing secondary leukemia such as acute myeloid leukemia over time. (126,127) MPN patients also are known to

have a high risk for arterial and venous thrombosis, and bleeding disorders (128–130). Even though the overall survival rate is higher than acute leukemias, the extent of the symptoms reduces quality of life, along with the higher risk of acquiring more aggressive secondary leukemias, prompted the need for understanding MPN pathogenesis and targeted therapies. Somatic JAK2 (JAK2V617F and JAK2 exon 12), MPL and CALR activating mutations are the most common driver mutations identified in the classical MPNs (123). Among these, JAK2-V617F mutations are observed in over 50% of MPN patients, with it being an almost exclusive mutation at 95% in PV **(Figure 1.5)**.

### 1.6.1. Role of MPN-stem cells in disease initiation and progression

A number of studies have suggested that the cell of origin for MPN-associated mutation acquisition are the HSCs. For example, the JAK2V617F mutation in MPN patients are observed in both the CD34+ CD38- stem and progenitor cells and in the mature cell lineages, suggesting that the mutation was acquired at the HSC or progenitor level in the hierarchy (131–133). Similar observations of mutation acquisition by the HSCs, sitting at the apex of the hematopoietic hierarchy, has also been reported in CALR mutated MPN patients (134). The presence of the mutation in the HSCs is not just required for MPN-initiation but is also required for disease propagation. This has been shown in JAK2 mutant mouse models, where only mutant HSCs and not the other multipotent progenitors, are capable of sustaining MPN-phenotype over a longer period of time (135). This would indicate that the acquisition of mutation by HSCs has to be followed by an expansion of the mutant clone due to a competitive advantage over genotypically normal HSCs, which subsequently leads to a MPN disease phenotype due to an eventual skew in mature cell production. This is supported by the observations that JAK2 mutations are frequently observed in individuals with clonal hematopoiesis (101). Furthermore, mutations in MPL and CALR are also associated with hypersensitivity to thrombopoietin (TPO), which is an important cytokine involved in HSC self-renewal (125,136,137). These findings further validate the clonal expansion of mutant HSCs as an important event in MPN pathogenesis. This is in contrast to other leukemias such as acute myeloid leukemia (AML), where transforming mutations in more lineage committed progenitors can also initiate and sustain the disease (138,139). Taken together, this highlights the importance of MPN-stem cells in not just disease initiation and propagation, but also

the importance of targeting this cell population for successful therapeutic interventions.

### 1.6.2. JAK2 mutations in MPN pathogenesis

JAK2 belongs to the Janus kinase family of tyrosine kinase proteins that sit downstream to important cytokine receptors. Cytokines that are known to be important mediators of hematopoiesis such as erythropoietin (EPO), thrombopoietin (TPO), granulocyte-macrophage colony stimulating factor (GM-CSF), Interleukin-3, etc., elicit their effects through the JAK/STAT signaling cascade (136,140–142). EPO and TPO are important mediators of erythropoiesis and megakaryopoiesis respectively, implicating the mutations in JAK2 such as the common JAK2V617F in PV and ET pathogenesis. JAK2V617F mutation is a point mutation leading to amino-acid substitution of valine to phenylalanine at position 617 of the amino acid chain. This leads to the constitutive activation of JAK2 signaling pathway, due to an altered pseudokinase domain of JAK2 (143) **(Figure 1.6)**. The change in the conformation of the pseudokinase domain is suspected to either limit its inhibitory action on the catalytic domain, or increase the kinase activity of the catalytic domain directly.

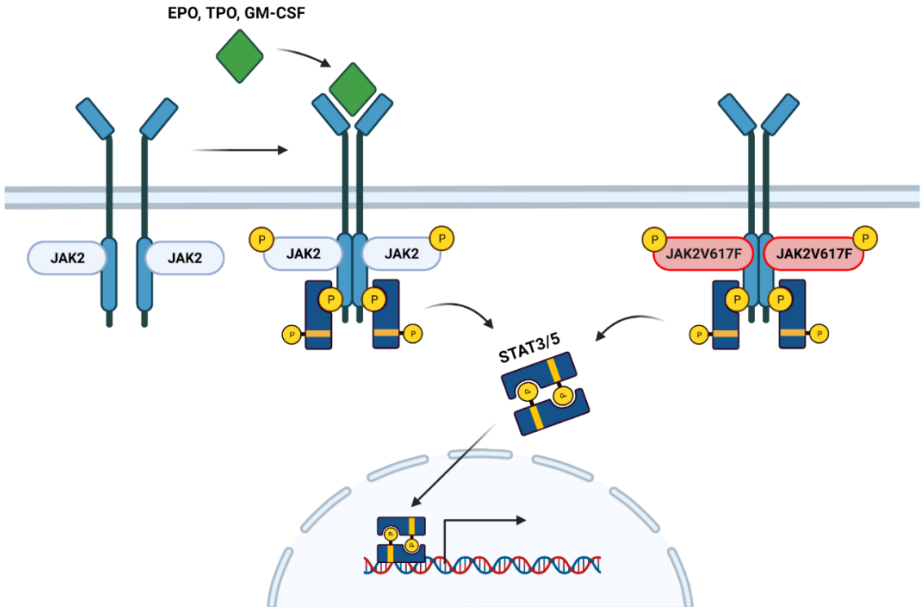


**Figure 1.6: JAK2V617F mutation leads to constitutive activation of the JAK-STAT signaling cascade**
*(Image created with Biorender.com)*

The varying disease phenotypes in PV and ET, even though driven by the same JAK2V617F mutation, suggests a role for the expression levels of mutant JAK2 and disease outcome. Individuals who are homozygous for JAK2V617F and have a high variant-allele frequency (VAF) are observed to tend to exhibit PV phenotype, and heterozygosity for the mutation with a low VAF tend to progress into an ET phenotype (144,145). However, there is a degree heterogeneity of disease outcomes within these groups of mutation status. The other JAK2 mutation is the JAK2 exon 12 mutation, which is exclusive to PV patients since the mutation leads to constitutive EPO receptor signaling and erythrocytosis (146). Other common mutations in JAK2V617F negative MPN patients include CALR and MPL mutations, that lead to constitutive MPL receptor activation and the subsequent JAK/STAT signaling (134,147). The JAK/STAT signaling sits at the point of convergence in the pathogenesis of the different mutations associated with MPNs and has hence been a prime target for therapeutic interventions in MPNs.

### 1.6.3. Treatment modalities for classic MPNs

The therapeutic options used for MPN patients are largely based on the subtype of MPN, prognosis and risk stratification, and the manifestation of disease-specific symptoms. Historically the symptoms in patients have driven the first line of therapy. The increased risk of thrombosis in PV and ET patients often leads to the use of low-dose aspirin, phlebotomy and cytoreductive therapy with hydroxyurea as the frontline therapy for these patients (148,149). Splenectomy is considered in patients of all MPN subtypes when they exhibit splenomegaly. The treatment guidelines are also based on the risk assessment of patients based on the International Prognostic Scoring System (IPSS) (150). Patients who are categorized as intermediate-2 or high-risk in the IPSS system can receive allogenic stem cell transplants. This is the only option that is completely curative for MPN patients with restoration of normal hematopoiesis, especially in PMF patients (151,152). However, the morbidity associated to the procedure, due to graft rejection and transplant-induced toxicities, limits its use to a narrow spectrum of patients (149). The identification of JAK2 mutations and the involvement of JAK/STAT pathways even in non-JAK2 mutations has led to the extensive use of JAK inhibitors in treating MPN patients. The most common JAK inhibitor that has been FDA- and EMA-approved and is frequently used in MPNs is Ruxolitinib. Ruxolitinib has been shown to have effective hematological response by

reducing blood hematocrit and splenomegaly in PV and ET patients, who were non-responsive to hydroxyurea treatment (153). The other JAK inhibitor approved for use in MPNs is Fedratinib, which is more specific in inhibiting JAK2 over the other JAK proteins (154). Even though JAK inhibitors have been found to be very effective in eliciting a hematological response in patients by normalizing the blood counts and reducing splenomegaly, both Ruxolitinib and Fedratinib administration does not lead to a molecular response in reducing the mutant allelic burden in patients (155,156). Interferon-α (IFNα) therapy is often used as an alternative to hydroxyurea for cytoreductive therapy for all MPN subtypes, and is the only option that is known to reduce the mutant allelic burden in patients (157–160).

### 1.6.4. Interferon-α as a therapeutic option in MPN

Interferon-α (IFNα) belongs to the type I interferon family of cytokines and has a multitude of functions such as anti-viral activity, immunomodulation, and pro- and anti-inflammatory effects (161–163). It is even attributed to having a beneficiary function in HSC specification and function during embryo development and embryonic hematopoiesis (50). Among the different isoforms of interferon-α, IFNα-2a and IFNα-2b are the isoforms that are widely used as therapeutic agents. The anti-viral activity of IFNα enabled its use to treat and manage viral infections such as hepatitis B and hepatitis C, and Kaposi sarcoma. Since the FDA approval in 1986 to the use of IFNα in treating hairy cell leukemia, many studies have validated the efficacy of IFNα in treating MPN patients. The rationale behind the use of IFNα for treating MPNs was based on its anti-proliferative effect on hematopoietic progenitor cells. *In vitro* and *in vivo* studies had shown that IFNα can significantly reduce the colony forming potential of erythroid, megakaryocytic and granulocytic progenitors in PV and PMF (164–166). Certain studies even showed that IFNα might have a selective effect on the MPN progenitor clones as opposed to healthy progenitors (167). Clinical trials of PV and ET patients treated with standard IFNα has shown to have a hematological response in 80% of patients and a complete molecular response in a significant proportion of those individuals by reducing the mutant allelic burden (168) **(Figure 1.7)**. IFNα treatment is also associated with an effective reduction in other MPN-associated symptoms such as splenomegaly, paresthesia, erythromelalgia, thrombosis, etc. (169)

Even though IFNα has been proven to be highly efficacious in treating all MPN subtypes, its use in clinics is still greatly limited. This is due to the fact that there is a high discontinuation rate of well over 20% in MPN patients receiving IFNα therapy (170). The high discontinuation rates are attributed to the adverse side effects faced by the patients to IFNα therapy. These include flu-like symptoms, hematological toxicity, elevated transaminases, nausea and severe fatigue (171). These issues led to the recent developments of adding polyethyleneglycol tail to IFNα, to generate pegylated-IFNα and ropegylated-IFNα. The addition of the polyethylene-glycol tail was done to enhance the half-life of IFNα in the blood plasma by increasing drug stability, without changing its therapeutic activity (172). Pegylated-IFNα alleviated the toxicity limitations of standard IFNα to an extent, due to the reduced frequency of administration of the compound (once per week as opposed to once per day with standard IFNα). However, even in clinical trials with pegylated-IFNα, patients still exhibited severe grade III/IV toxicity that led to a significant discontinuation rate of 20% (173).

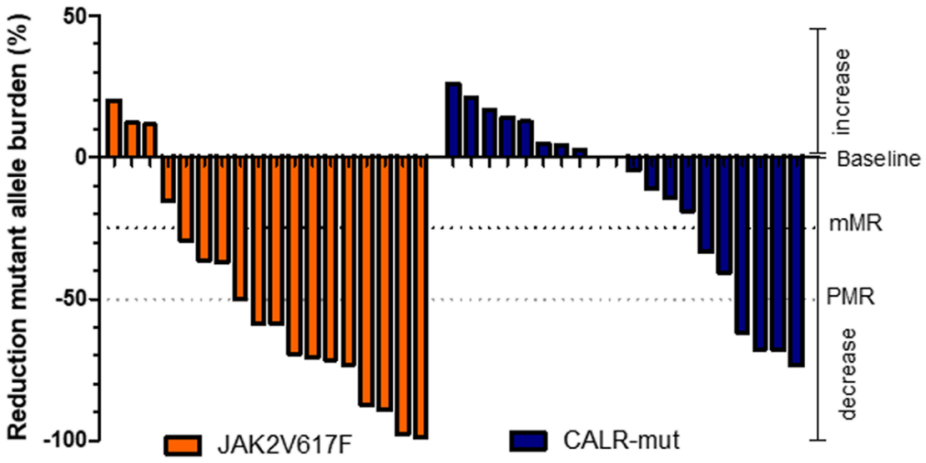


**Figure 1.7: IFNα therapy leads to reduction in mutant allelic burden in MPN patients with JAK2V617F or CALR mutations**
*(Image from* (174)*; Reprinted with permission from Springer Nature through copyright.com)*

Any potential benefits of IFNα in effectively treating MPNs is greatly hampered by the adverse side effects. This required the understanding of how IFNα works in achieving molecular remission in patients to either find alternative therapies, or to further sensitize patients to IFNα treatment to broaden the therapeutic window.

However, the exact mechanism of action of IFNα in reducing the mutant allelic burden is not yet completely understood. With the previously mentioned importance of MPN stem cells in disease initiation and progression, one suggested mode of action of IFNα is by eliminating the mutant stem cells. This hypothesis was based on the observations that IFNα has been shown to push genotypically normal dormant HSCs in mouse bone marrow into active proliferation, and the chronic treatment leads to attrition of HSC function (27). A study performed on a JAK2V617F mutant mouse model has shown that IFNα treatment leads to a selective depletion of the mutant HSCs in the bone marrow, supporting this hypothesis (175). Furthermore, it has been shown that JAK2 mutant HSCs are particularly sensitive to DNA damage acquisition upon treatment with pegylated-IFNα in a mouse model of PV (176). This is in line with previous observations that the attrition of function in wildtype HSCs upon chronic IFNα treatment is associated to acquisition of replication-stress induced DNA damage. However, whether this depletion of mutant HSCs is specific to IFNα therapy and if the lack of molecular response to JAK inhibitors is due to an inherent dormancy of mutant HSCs in the bone marrow, are questions that haven't been explored in detail. These studies would not only enable better understanding of the mode of action of IFNα therapy in relation to existing first-line therapies, but also enable a better understanding of the role of the disease propagating MPN stem cells in therapy resistance. This would further lead to better targeting for therapeutic interventions while bypassing or reducing IFNα-associated toxicities for MPN patients.

# 2 Aims of the Thesis

The continued production of diverse mature cells present in the peripheral blood is of paramount importance to the survival of any organism. The hematopoietic stem cells (HSCs) that reside in the bone marrow is of vital importance to this process, both under homeostasis and under stress-induced hematopoiesis. This exemplifies HSCs as some of the most important cells in an individual and its functional preservation as a key factor for survival. The role of infections and inflammatory cytokines such as IFNα on hematopoiesis and hematological neoplasms is of particular interest due to their direct effects of HSC biology. The work in this thesis looked into different aspects of this and has been divided into two parts – IFNα as a therapeutic agent for myeloid neoplasms, and infections as a mediator of stress-hematopoiesis.

**Part-one** of this dissertation involves the work to elucidate the mechanism of action of IFNα therapy in the treatment of myeloproliferative neoplasms (MPN). The majority of patients with Bcr-Abl negative MPN have a JAK2V617F mutation (177). Therapeutic options for JAK2 mutated MPNs include targeted therapy with JAK-inhibitors such as Fedratinib and interferon-alpha (IFNα) therapy. Among these IFNα therapy is the only option that leads to a molecular response in patients (155,174). However, patients frequently exhibit adverse side-effects to IFNα treatment (173). This also applies to pegylated forms of IFNα. Even though peg-IFNα can be almost curative for MPN patients, the exact mechanism of how it works in achieving mutant allelic burden reduction, is not yet completely understood and requires further studies. To this end, the following were the main aims of this part of the thesis:

- Assess the inherent proliferative index of the disease-initiating JAK2 mutant MPN-stem cells
- Identify whether a dormant fraction of JAK2 mutant MPN-stem cells exist in the bone marrow

- Evaluate the differential effects of JAK inhibitor (Fedratinib) treatment and pegylated-IFNα treatment on MPN-associated symptoms and mutant allelic burden
- Assess the effect of pegylated-IFNα treatment on the mutant MPN-stem cells, as a potential mechanism of action

**Part-two** of the dissertation deals with studies performed to assess co-housing as a model system to study the effect of infections on hematopoiesis and HSCs. Previously used mouse models to study the effects of infections on hematopoiesis have all shown that chronic infections in mice leads to increased proliferation of HSCs and a loss of functional potency (28,30,76,77). However, the major caveat with all of these models is that they either involve injecting mice with a compound that only mimics an infection or involve injecting mice with non-physiologically high dose of a purified pathogen. This does not recapitulate a natural process of infection initiation or response in humans. A co-housing model of infection as previously described by Beura *et al*. (84), but performed with wilding mice (89), was hence hypothesized to be a more natural method to study bone marrow hematopoiesis upon infection-response. Moreover, the timing of acquisition of infections in mice (as an adult versus neonatal exposure), and its differential effect on hematopoiesis, remains an unexplored question. Hence, the following were the main aims of the second part of this thesis:

- Evaluate whether co-housing laboratory mice with wildling mice leads to efficient transmission of infections
- Assess the acute effects of co-housing mediated infections on peripheral blood counts, bone marrow hematopoiesis and spleen hematopoiesis
- Assess the chronic effects of co-housing mediated infections on peripheral blood counts, bone marrow hematopoiesis and spleen hematopoiesis
- Compare the effects of co-housing mediated horizontal transmission of infections against neonatal vertical transmission of infections in wildling mice
- Study the effects of acquiring infections as an adult and acquiring infections neonatally, on HSC dormancy and functional potency
- Validate the use of co-housing model of infections to study mouse-strain dependent effects of infection on hematopoiesis
- Validate the use of co-housing model to understand the effects of infections on clonal hematopoiesis in mice

# 3 Results - I

## Mechanism of action of Interferon alpha therapy in the treatment of myeloproliferative neoplasms

Interferon-alpha (IFNα) is the only therapeutic option available for patients with Jak2V617F-mutant myeloproliferative neosplams (MPN) that can lead to a significant reduction in the mutant allelic burden (174). However, IFNα treatment is accompanied by adverse side effects and many patients drop out of treatment just because of these (173). Even though IFNα can be highly efficacious in treating MPN patients, the exact mechanism of how it works in achieving molecular remission in patients, is not yet completely known. Hence in this part of the thesis, I aimed to understand the mechanism of action specific to IFNα treatment in treating MPNs.

## 3.1. Establishing a transplantation-based Jak2V617F mouse model and characterization of MPN development

In order to understand the mode of action of IFNα against Jak2 mutated MPNs, I first needed a suitable model system that would mimic human MPNs. For this, a previously published mouse model of Jak2V617F mutation (178) was employed to generate chimeric mice with 30% Jak2 mutant cells in the bone marrow **(Figure 3.1A)**. Cre recombinase inducible Jak2V617F transgenic mice were crossed with Tamoxifen-inducible Rosa26-CreERT mice. Bone marrow from the resultant transgenic mouse was transplanted into lethally irradiated recipient mice along with wildtype (WT) bone marrow in a 1:2 (V617F:WT) ratio. The proportion of Jak2 mutant cells was kept lower to mimic myeloproliferative neoplasms in humans, where only a subset of the bone marrow cells carry the mutation. The two donor mice (Jak2V617F or RosaCre and wildtype competitor) and the recipient mice express different CD45 isoforms, namely CD45.1, CD45.1/2 and CD45.2. Hence the different donor bone

marrow cells can be distinguished based on the surface CD45 isoform when analyzed suing flow cytometry. The generated chimeric mice (referred to as Jak2-mutant mice hereafter) were then administered with Tamoxifen, two months post-transplantation, to activate the Cre recombinase and induce the Jak2V617F mutation. Mice transplanted with bone marrow from Rosa-CreERT mice were used as control mice in the experiment to account for any Cre or Tamoxifen mediated effects.

### 3.1.1. Mice with Jak2 mutant bone marrow showed elevated peripheral blood cell counts mimicking polycythemia Vera

Blood samples were collected from the transplanted chimeric mice once every 4 weeks to assess disease progression. Jak2 mutant mice exhibited elevated white cell count (WCC), red cell count (RCC), hemoglobin (Hb) and hematocrit (HCT) starting from 10 weeks after Tamoxifen administration **(Figure 3.1B,C,D,E)**. The RCC, HCT and Hb values continued to increase over a period of 18 weeks as the disease progressed. The observed increase in the peripheral blood parameters are similar to that observed in human patients with polycythemia Vera. These changes have also been reported in the primary non-transplanted Jak2V617F mouse model. It is to be noted that even a lower percentage of mutant cells in the bone marrow of these chimeric mice leads to a similar disease progression as observed in the primary mutant mice with very high proportions of mutant bone marrow cells, albeit with a slower disease initiation.

### 3.1.2. Jak2V617F mutant mice exhibited splenomegaly similar to that of human polycythemia Vera

The Jak2 mutant mice were observed to develop splenomegaly compared to the Cre control mice **(Figure 3.2A)**. I observed that the spleen weight continued to increase over the course of disease progression in the Jak2 mutant mice and this was also concomitant with an increase in the number of white blood cells in the spleen **(Figure 3.2B)**. Since splenomegaly in polycythemia vera (PV) could potentially be attributed to altered erythropoiesis in the spleen, I also assessed the maturation of erythrocytes in the spleen using flow cytometry **(Figure 3.2C)**. CD71 and Ter119 double-positive cells are defined as immature erythrocytes and the Ter119 single-positive cells are defined as mature erythrocytes. I observed an accumulation of

immature erythroid cells in the spleen of Jak2 mutant mice starting from six weeks post induction of the mutation with Tamoxifen **(Figure 3.2D & E)**. Concomitantly there was a decrease in the proportion of mature erythrocytes. This suggests that there is an increased demand of erythropoiesis in the spleen and hence an accumulation of the immature erythrocytes. All in all, the increased hematocrit and splenomegaly demonstrates that the mouse model mimics human polycythemia vera, and hence serves as a suitable model system to further assess the disease and treatment modalities.

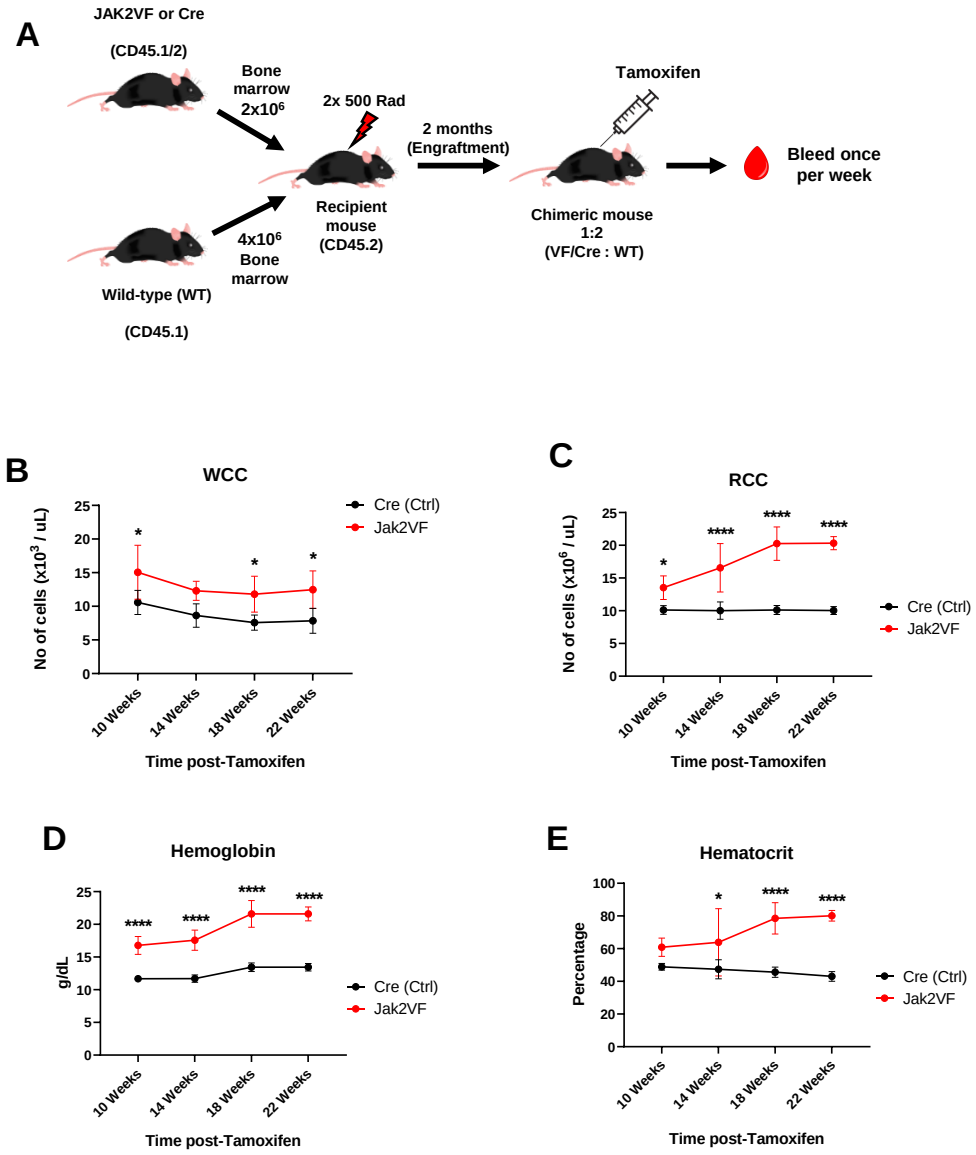


Figure 3.1: Jak2V617F mutant mice show elevated peripheral blood white cell counts and increasing hematocrit values.

**(A)** Experimental scheme to generate chimeric Jak2 mutant mice through bone marrow transplantation and study disease progression. **(B)** Changes in peripheral blood white cell count (WCC) from 10 weeks post-induction of mutation. **(C)** Changes in peripheral blood red cells count (RCC) from 10 weeks post-induction of mutation. **(D)** Changes in peripheral blood hemoglobin content from 10 weeks post-induction of mutation. **(E)** Changes in blood hematocrit percentage from 10 weeks post-induction of mutation.
*(n=4-6 per group; All values are mean±SD; * represents P<0.05 and **** represents P<0.0001)*

## 3.2. Jak2VF mutant HSCs have increased proliferation on average but a dormant subpopulation of mutant HSCs exists in the bone marrow

The existence of dormant populations of cancer stem cells and their role in disease propagation and therapy resistance have been widely speculated by many (179–183). The dormant nature of such cells could be a prime mechanism of evading standard chemotherapy regimens as many of these drugs rely on cellular proliferation. However, conclusive data demonstrating their existence of such cells is still lacking. Many hematopoietic neoplasms including MPNs, being diseases driven by mutations in the hematopoietic stem cells, serve as a suitable model to assess the existence of dormant cancer stem cell populations. Hence, I first assessed the proliferative index of Jak2 mutant HSCs during disease progression. To this end, a classical BrdU pulse-chase experiment was performed on Jak2 mutant mice.

I generated chimeric Jak2 mutant mice or Cre control mice in a 1:2 ratio (VF/Cre:WT) as previously described **(Figure 3.1A)** and then induced the Jak2 mutation through Tamoxifen administration. Following Tamoxifen treatment, the mice were administered with BrdU (0.8mg/mL) through drinking water for a period of 14 days to pulse label cells. The BrdU administration was then stopped to start the chase period. Cohorts of mice were euthanized and analyzed at different chase time-points **(Figure 3.2A)**. The proportion of BrdU-positive HSCs were assessed using flow cytometry **(Figure 3.3B)**. BrdU-positive HSCs here would hence represent dormant HSCs that have not divided and hence have not lost BrdU incorporated in the DNA, while BrdU negative HSCs represent HSCs that have actively divided in the bone marrow.

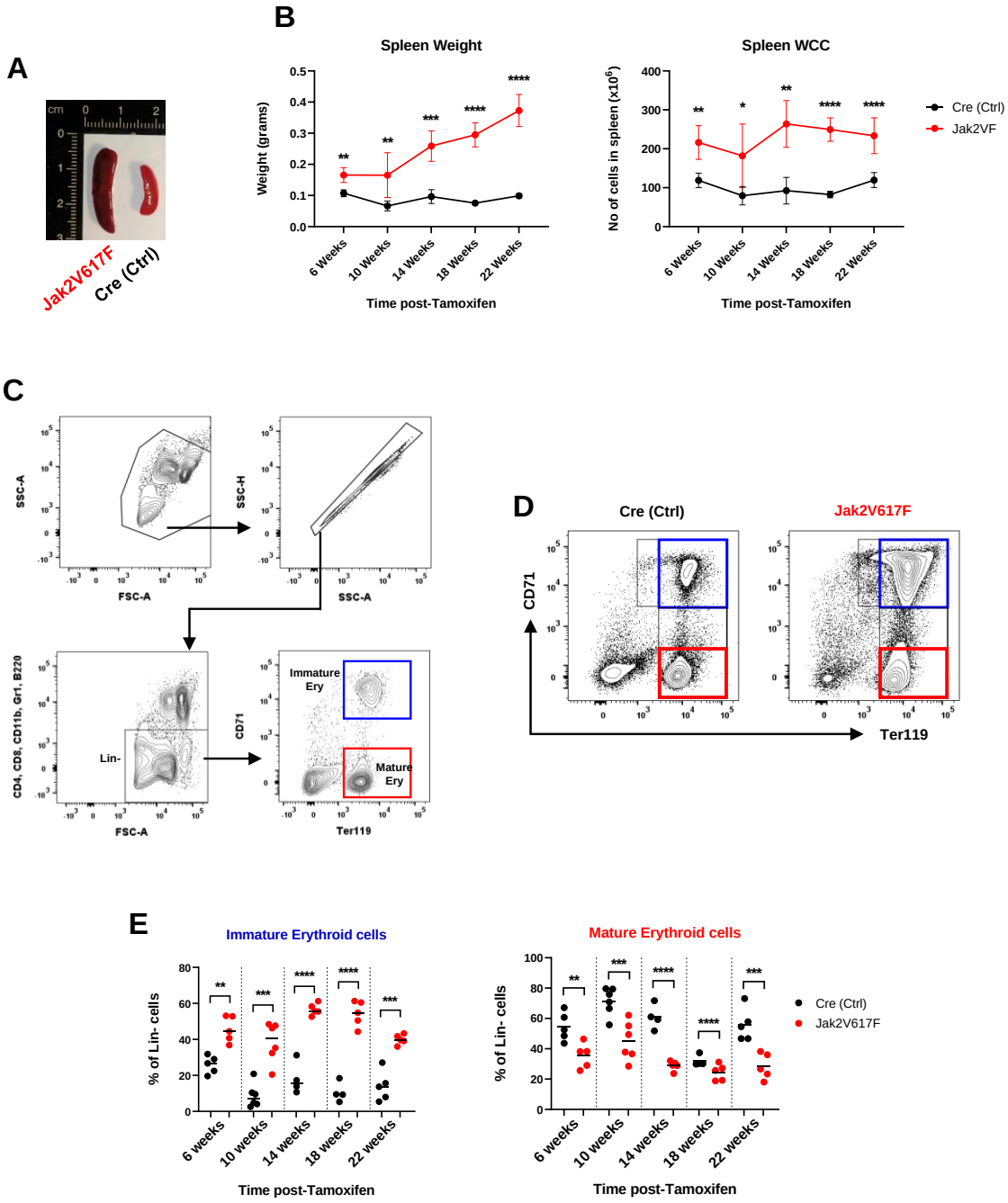


**Figure 3.2: Jak2V617F mutant mice exhibit splenomegaly and accumulation of immature erythrocytes in the spleen.**

**(A)** Representative spleens of Jak2 mutant and Cre control mice at 14 weeks post-Tamoxifen administration. **(B)** Spleen weights of Jak2VF and Cre control mice sacrificed at different time-points post-Tamoxifen administration. **(C)** Flow cytometry gating scheme for immature erythrocytes (blue box) and mature erythrocytes (red box). **(D)** Representative contour plots showing immature and mature erythrocytes in the spleen of Jak2VF and Cre control mice. **(E)** Percentage of immature and mature erythrocytes in the spleen of Jak2VF and Cre control mice at different time-points post-Tamoxifen.

*(n=4-6 per group; All values are mean±SD; * represents P<0.05, ** represents P<0.01, *** represents P<0.001 and **** represents P<0.0001)*

The Jak2 mutant HSCs showed a significantly faster BrdU label-dilution compared to WT Cre HSCs **(Figure 3.3C)**. This shows that the Jak2 mutant HSCs have an increased proliferation compared to WT HSCs. However, I observed that even at 20 weeks of chase, there still existed BrdU-high Jak2 mutant HSCs in the bone marrow **(Figure 3.3D)**. This suggests the existence of a subfraction of Jak2 mutant HSCs that stay dormant, even though on average the mutant HSCs have increased proliferation. Hence this finding identifies a novel sub-fraction of mutant cancer stem cells that are dormant, as has been previously speculated in many cancer entities (179–183). These cells could effectively be driving the disease and offering resistance to standard therapies such as cytoreductive therapies and JAK inhibitor therapies. However, it has to be noted here that the initial BrdU labelling efficiency was only around 80% in the HSC compartment. The remaining 20% mutant HSCs that did not incorporate BrdU could be more dormant mutant HSCs that were not labelled with BrdU. Hence, the actual percentage of dormant Jak2 mutant HSC fraction might be even larger than what is observed here. An initial labelling mechanism which is independent of proliferation would be needed here to get a more accurate picture of the true proportion of dormant disease propagating HSCs.

## 3.3. Comparing and contrasting the effect of Fedratinib (JAK inhibitor) and pegylated IFN-α on polycythemia Vera

JAK inhibitors (Fedratinib and Ruxolitinib) and IFNα include the standard treatment options for treating patients with Jak2 mutant MPNs such as polycythemia vera. Among them, only IFNα is capable of achieving molecular remission in patients, while JAK inhibitors only lead to hematological response with a normalization of blood parameters (155,156,174). Based on existing literature, it is known that IFNα treatment of wildtype mice pushes dormant HSCs to proliferate and chronic treatments have been shown to deplete functional HSCs in the bone marrow due to replication stress. Having seen that a dormant fraction of Jak2 mutant HSCs persist in the bone marrow of mice with MPN, it was postulated that repeatedly pushing the dormant Jak2 mutant HSCs into replication and thereby eliminating them in the bone marrow is how IFNα is able to achieve molecular remission in patients. JAK inhibitors on the other hand might not be eliciting an effect on these dormant mutant HSCs. Hence to compare the effects of pegylated-IFNα (peg-IFNα) and the JAK inhibitor

Fedratinib on the dormant mutant HSC fraction that I previously identified, I performed a BrdU pulse-chase experiment while treating mutant mice with these drugs.

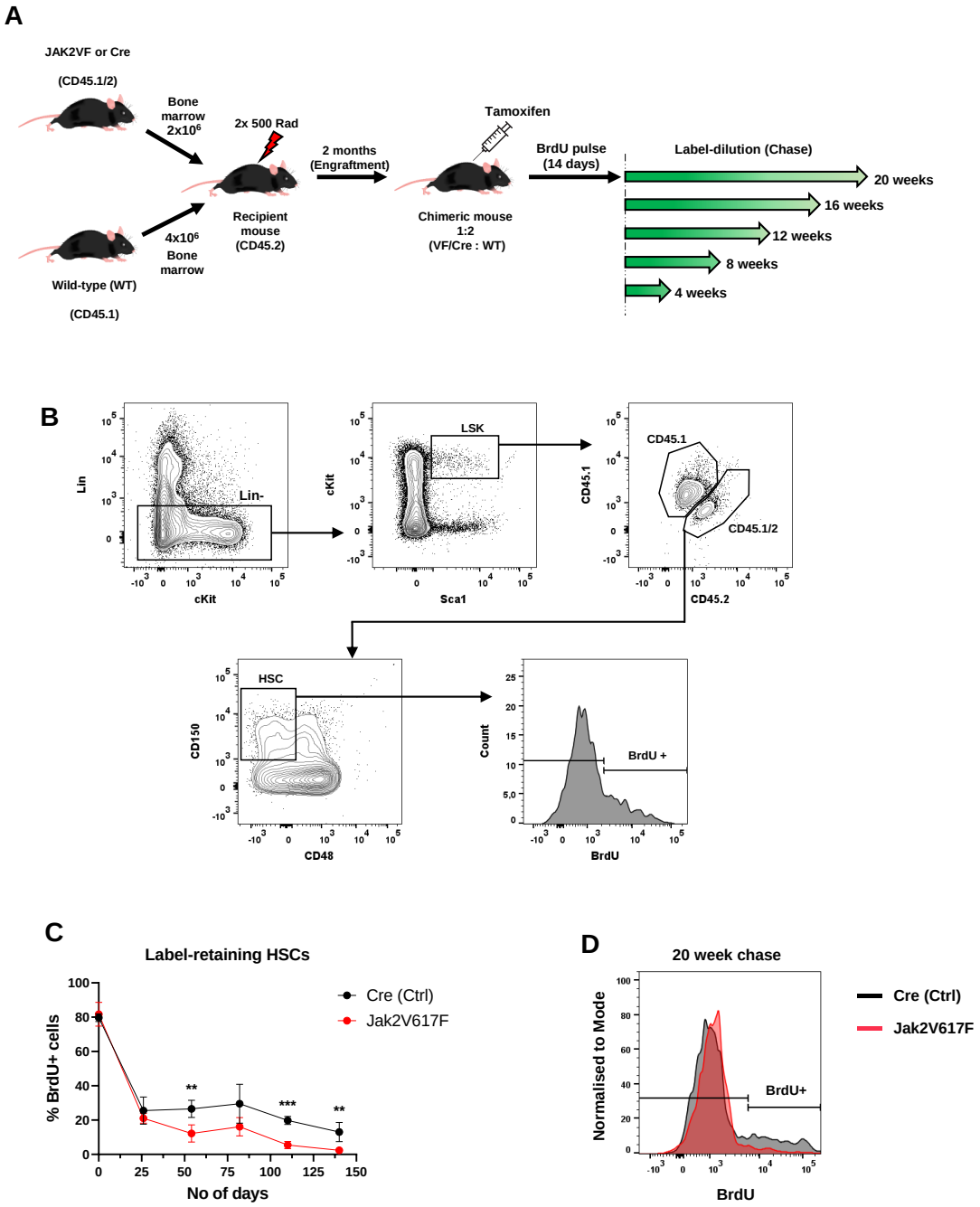


**Figure 3.3: BrdU pulse-chase reveals Jak2 mutant HSCs proliferate more on average but a fraction of mutant HSCs stay dormant in the bone marrow**
**(A)** Experimental scheme for BrdU pulse-chase experiment to assess the proliferative index of Jak2 mutant HSCs compared to Cre control HSCs. **(B)** Flow cytometry gating scheme to measure the

proportion of BrdU+ label-retaining HSCs in the bone marrow. **(C)** Proportion of BrdU+ label-retaining (dormant) HSCs in the bone marrow of Jak2VF and control mice euthanized at different chase time-points. **(D)** Representative histogram showing the proportion of BrdU+ HSCs at 20 weeks of chase in the Jak2VF and control mice bone marrow.
*(n=4-6 per group; All values are mean±SD; ** represents P<0.01 and *** represents P<0.001)*

Chimeric Jak2 mutant mice were generated through bone marrow transplantation as described in previous sections in a 1:2 ratio (VF:WT). The Jak2 mutation was induced by administering Tamoxifen to the mice. The mice were then administered a 14 day BrdU-pulse through drinking water after a period of 10 weeks from the induction of the mutation. Two weeks after the BrdU pulse, the mice were administered with either pegylated-IFNα (300ng or 600ng), or with Fedratinib (120mg/kg BW) for a period of 4 weeks. **(Figure 3.4A)** 600ng dosage of peg-IFNα was recommended by Pharmaessentia who provided us the murine peg-IFNα based on their analysis of efficacy testing. I additionally assessed the lower dosage of 300ng to understand the degree to which the efficacy changes in terms of PV symptoms and effect on dormant mutant HSCs. Mice treated with the respective drug vehicles (0.1% BSA for peg-IFNα, and 45% PBS + 10% DMSO + 40% PEG400 + 5% Tween-80 for Fedratinib) were used as control mice for the experiment. The peg-IFNα was administered once per week for the duration of the treatment and Fedratinib was administered 5 days per week for the stipulated treatment period. The mice were euthanized after the 4 weeks treatment regimen to analyze the spleen and the proportion of BrdU+ dormant HSCs in the bone marrow. Blood samples were also collected before and after the drug treatment to assess pre- and post-treatment blood parameters and mutant allelic burden.

### 3.3.1. Both Fedratinib and pegylated IFN-α are capable of alleviating the symptoms associated with PV in mice

The Jak2 mutant mouse model exhibited three symptoms associated with PV – elevated Hematocrit, splenomegaly and accumulation of immature erythrocytes in the spleen. Hence, I first evaluated the effects elicited by Fedratinib and IFNα on these PV symptoms. The mice treated with the respective vehicles for Fedratinib and IFNα showed increasing hematocrit values during the treatment period, indicating continued disease progression in these mice **(Figure 3.4B)**. However, the Fedratinib

treated mice and the 300ng peg-IFNα treated mice did not show further significant increase in hematocrit, suggesting that the treatment stabilized this value by reducing further disease progression. The dosage used might not have been sufficient to reduce the red cell count as erythrocytes have a long half-life (184). 600ng dosage of peg-IFNα on the other hand was able to significantly reduce the hematocrit value to normal levels. In conclusion, both Fedratinib and peg-IFNα was capable of reducing, or at least stabilizing, the peripheral blood hematocrit.

I next assessed the effect of the drugs on spleen-associated symptoms. Both Fedratinib and higher dose of peg-IFNα were able to effectively treat splenomegaly with a significant reduction in the spleen weights compared to the respective vehicle controls **(Figure 3.4C)**. The 300ng peg-IFNα treatment did not show a significant reduction in spleen weight but there was a trend towards reduced spleen weight. I then analyzed the splenocytes by flow cytometry to assess the proportion of immature erythrocytes in the spleen. Concomitant to spleen weights, the proportion of immature erythrocytes in the spleen was also significantly reduced by both Fedratinib and peg-IFNα treatments compared to the vehicle controls **(Figure 3.4D & E)**. The 600ng dosage of peg-IFNα was however observed to be the most effective in the reduction of accumulated immature erythrocytes in spleen.

In conclusion, these results suggest that both Fedratinib and peg-IFNα are capable of alleviating the symptoms associated with PV. This is similar to what is normally observed in human PV patients, where both these treatment options led to a normalization of the blood parameters and reduction in splenomegaly. Thus the data from the mouse model recapitulates the overall effect of these two treatment regiments in patients.

### 3.3.2. Only peg-IFNα stabilizes the mutant allelic burden in Jak2 mutant mice

In human patients with PV, it is known that IFNα is the only treatment that leads to a reduction in the mutant allelic burden (160). Hence, I also assessed the peripheral blood of the mutant mice following Fedratinib and peg-IFNα treatments to compare them. The Jak2 mutant cells transplanted in this model should express the surface marker CD45.1 and can be distinguished from the wildtype CD45.1/2 expressing cells in the recipient mice. Hence the proportion of Jak2 mutant white blood

cells measured in the peripheral blood can be considered as a representative value of the mutant allelic burden.

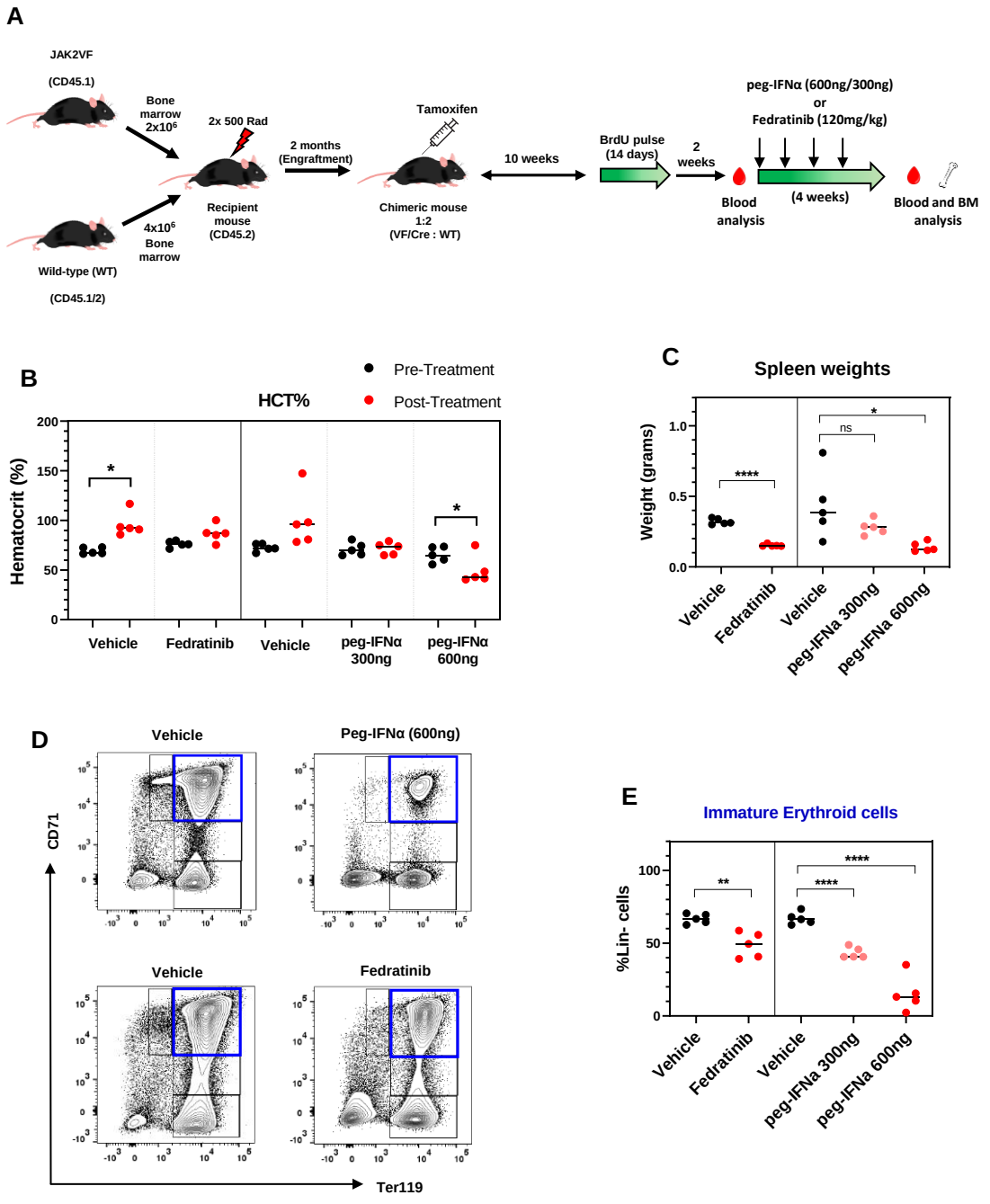


**Figure 3.4: Both Fedratinib and peg-IFNα are capable of alleviating the symptoms associated with PV**

**(A)** Experimental scheme to compare the effects of Fedratinib and peg-IFNα treatments on PV symptoms and on the dormant BrdU label-retaining mutant HSCs. **(B)** Pre- and post-treatment peripheral blood hematocrit percentages among different treatment groups. **(C)** Post-treatment spleen weights of mice among different treatment groups. **(D)** Representative contour plots showing

immature erythrocytes (blue gate) in the spleen of mice among different treatment groups. **(E)** Percentage of immature erythrocytes in the spleen of mice among different treatment groups.
*(n=5 per group; All values are mean±SD; * represents P<0.05, ** represents P<0.01, **** represents P<0.0001 and ns represents non-significant)*

I observed that the mice treated with the respective vehicles for Fedratinib and peg-IFNα showed a continued increase in the proportion of CD45.1 mutant cells in the peripheral blood **(Figure 3.5)**. This shows continued progression of the disease as expected. However, the Fedratinib treated mice also showed an increase in mutant cells in peripheral blood suggesting that Fedratinib has no effect on the mutant allelic burden. Both doses of peg-IFNα on the other hand were able to prevent the increase in proportion of mutant cells in the peripheral blood suggesting a stabilization of the mutant allelic burden. The 600ng dosage of peg-IFNα was even capable of reducing the proportion of mutant cells in some of the mice **(Figure 3.5)**. These results again reiterate what is seen in human MPN patients, wherein only peg-IFNα can lead to a reduction in the mutant allelic burden while Fedratinib cannot achieve this (185).

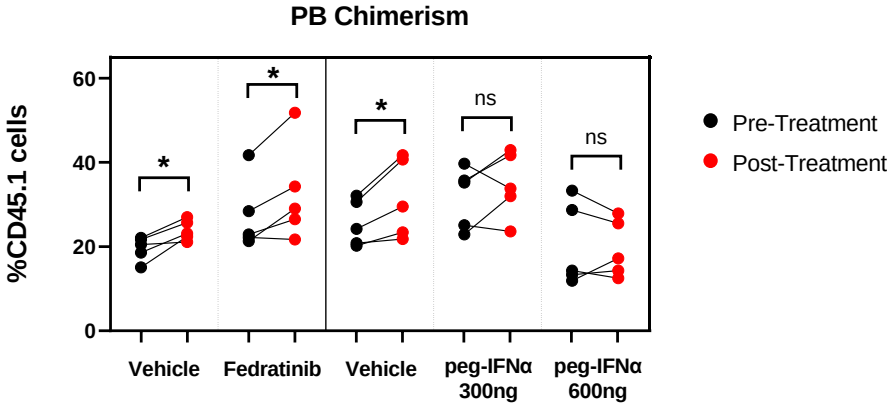


**Figure 3.5: Only pegylated IFNa stabilizes the peripheral blood mutant allelic burden**
Pre- and post-treatment percentages of CD45.1 Jak2 mutant cells in the peripheral blood of mice among different treatment groups
*(n=5 per group; All values are mean±SD; * represents P<0.05 and ns represents non-significant)*

### 3.3.3. Peg-IFNα pushes dormant Jak2 mutant HSCs into proliferation and reduces the frequency of mutant HSCs in the bone marrow while Fedratinib elicits no effect on mutant HSCs

As I observed that peg-IFNα was able to stabilize the proportion of mutant cells in the peripheral blood, I next wanted to assess whether this is due to an effect on the mutant HSCs in the bone marrow. To this end, the frequency of mutant HSCs in the bone marrow was measured, post-treatment with Fedratinib or peg-IFNα. It was observed that only the 600ng peg-IFNα treatment led to a significant reduction in the frequency of mutant HSCs **(Figure 3.6A)**. In contrast, Fedratinib treatment did not result in a reduction in the frequency of mutant HSCs, suggesting that Fedratinib does not affect the mutant HSCs. This could be the main difference between Fedratinib and peg-IFNα treatments and why Fedratinib treatment is unable to reduce or stabilize the mutant allelic burden like peg-IFNα.

Knowing that only peg-IFNα eliminates the mutant HSCs in the bone marrow, I assessed the effect of the drugs on the dormant fraction of the Jak2 mutant HSCs. Following a BrdU pulse pre-treatment, the proportion of BrdU label-retaining dormant mutant HSCs was analyzed post-treatment using flow cytometry. First of all, Fedratinib did not lead to any reduction in the proportion of BrdU+ HSCs, suggesting that it does not elicit an effect of pushing the dormant mutant HSCs into proliferation **(Figure 3.6B & C)**. On the other hand, peg-IFNα treatment showed a trend of reduction of BrdU-positive dormant mutant HSCs. This indicates that peg-IFNα was capable of driving the dormant mutant HSCs in the bone marrow into cycle. This specific phenomenon of eliminating the dormant mutant HSCs is what distinguishes the effects of peg-IFNα versus Fedratinib. Hence this ability of peg-IFNα to push the dormant mutant HSC fraction into proliferation and subsequently eliminate them, might be the specific mechanism of how IFNα treatment achieves a reduction in the mutant allelic burden in patients. However, the data here is only a correlative assessment of the stabilization of peripheral blood mutant cells and the elimination of dormant mutant HSCs in the bone marrow. Further functional analysis of the dormant and cycling fractions of the mutant HSCs, in terms of their ability to propagate the disease in secondary transplants, is required to ascertain whether the elimination of dormancy in mutant HSCs has a direct causal relationship with mutant allelic burden reduction. Also, the lower dose of peg-IFNα at 300ng was not able to significantly reduce the frequency of mutant HSCs even though there was a reduction in the

proportion of dormant mutant HSCs **(Figure 3.6B & C)**. This suggests that a longer treatment regimen to induce further cycling of the mutant HSCs.

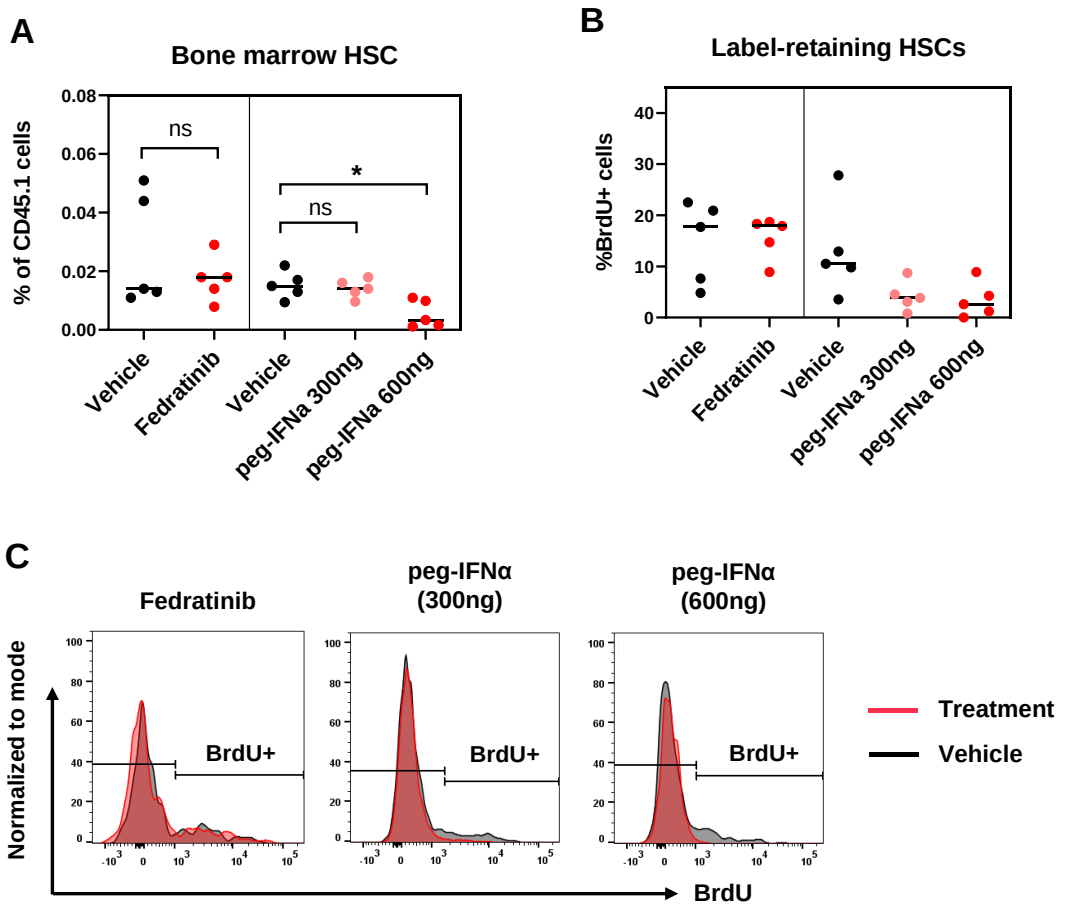


**Figure 3.6: Pegylated IFN-a depletes dormant Jak2 mutant HSCs in the bone marrow while Fedratinib has not effect on mutant HSCs**

**(A)** Post-treatment frequency of CD45.1 Jak2 mutant HSCs in the bone marrow of mice among different treatment groups. **(B)** Post treatment proportion of mutant HSCs that are BrdU+ (dormant) among the different treatment groups. **(C)** Representative histograms of BrdU+ mutant HSCs following treatment with Fedratinib or peg-IFNα compared to their respective vehicle controls.

*(n=5 per group; All values are mean±SD; * represents P<0.05 and ns represents non-significant)*

# 4 Discussion – I

## Mechanism of action of Interferon alpha therapy in the treatment of myeloproliferative neoplasms

The majority of patients with Bcr-Abl negative myeloproliferative neoplasms (MPN) have a JAK2-V617F mutation (177). Targeted therapy with JAK-inhibitors such as Ruxolitinib and Fedratinib has been shown to normalize blood parameters, but does not lead to a reduction in the mutant allelic burden (155,156). Interferon-alpha (IFNα) therapy is the only treatment option available for these patients that can achieve molecular remission with a significant reduction in the mutant allelic burden (174). However, long-term treatment with IFNα is required and patients frequently exhibit adverse side-effects (173). The advent of pegylated forms of IFNα (peg-IFNα) has reduced the drawbacks by reducing the frequency of injections needed by the patients, and reducing the severity of the side effects. Nevertheless, even peg-IFNα is still found to induce grade III/IV toxicity in patients leading to treatment stoppages and dropouts (173). Even though peg-IFNα can be curative for MPN patients, the exact mechanism of how it works in achieving mutant allelic burden reduction, is not yet completely known. Hence in this part of the thesis, I aimed to elucidate the mechanism of action of peg-IFNα treatment, to identify options to mitigate the adverse effects of the treatment. To understand the mechanism of action of a drug, it is first essential to understand the characters of the cells that initiate and propagate the disease.

### 4.1. MPN stem cell dormancy and potential therapy resistance

Mutant HSCs have been identified to be the main disease initiating population in Jak2 mutant MPNs in a mouse model of JAK2V617F-mutant polycythemia vera (PV) (135). It was shown that transplanting the mutant HSCs, and not other mutant progenitor cells such as MPPs, was required to initiate and sustain PV symptoms in

the recipient mice. This highlights the importance of the mutant HSCs or the MPN-stem cells in the disease and also points to a potential target for the success of a therapeutic modality in curing the disease and not just treating the symptoms. The importance of stem-cell-like cancer cells called cancer stem cells (CSC) have also been widely studied and speculated in other cancers including AML (179,180,186,187). These CSCs are not just important for disease initiation and propagation, but also could be potentially causing therapy resistance in patients. Some mechanisms through which cancer stem cells mediate therapy resistance have been previously reported and include drug efflux through transporters, immune evasion, defective DNA damage response, deregulation of apoptosis, dormancy, etc. (188) Among these, CSC dormancy was of a particular interest to me in relation to MPNs, since normal wildtype HSCs in the bone marrow are known to predominantly exist in a deeply dormant state (25). If the dormancy of normal HSCs is retained in mutant HSCs, this could prove to be an important mechanism of therapy resistance in MPNs.

### 4.1.1. A dormant sub fraction of Jak2 mutant MPN stem cells persist in the bone marrow

The existence of cancer stem cells that stay dormant in patients and their role in disease propagation and therapy resistance, has been constantly speculated in literature (179–183). The lack of continued proliferation in these cells could be an important mechanism of evading classic chemotherapies as many of these drugs target proliferating cells. However, conclusive evidence of existence of these dormant CSCs is still lacking. Most of the hematopoietic neoplasms including MPNs could serve as a suitable model to assess dormant CSC existence, since mutations at the stem cell level act as the main drivers of these malignancies. Existing data on CSC dormancy in myeloid malignancies have shown that the proportion of proliferating mutant HSCs increase following treatment with drugs that induce proliferation in HSCs, indirectly claiming the existence of dormant mutant HSCs in the bone marrow (175,189). Nevertheless, these studies have relied on the analysis of cell cycle phase of the mutant HSCs as a measure of dormancy. However, this only gives a snapshot of how many cells are proliferating at the specific time-point of measurement. They do not give any retrospective information as to whether a persistently dormant pool of mutant HSCs can exist. Hence, as part of my study, I performed a classical BrdU pulse-chase experiment over a period of 20 weeks in Jak2 mutant mice to assess the proliferative

index of the Jak2 mutant HSCs. This approach gave a measure of mutant HSC proliferation over time but also showed whether there are HSCs that have not divided during the period of observation.

Jak2 mutant HSCs were found to have increased proliferation as observed through a faster dilution of the BrdU label in the mutant HSCs. This is not surprising as Jak2 mutations are also known to be present in individuals with clonal hematopoiesis, where a subset of HSCs have a self-renewal and proliferative advantage (101). However, even at 20 weeks of chase, I observed the existence of some label-retaining dormant mutant HSCs. This indicates that a proportion of Jak2 mutant MPN-stem cells stay dormant during disease progression even though they proliferate more on average. This is a novel finding that definitively shows the existence of a sub-fraction of mutant cancer stem cells that are dormant, as has been previously speculated in many cancer entities. These cells could effectively be driving the disease and offering resistance to standard therapeutic options such as cytoreductive therapies and JAK inhibitor therapies. This also gave us the opportunity to assess if targeting the dormant HSCs is how peg-IFNα effectively achieves molecular remission.

## 4.2. IFNα specific changes in disease outcome in Jak2 mutant MPN points to its potential mechanism of action

As previously mentioned, peg-IFNα is so far the only therapeutic option that leads to a significant reduction in the mutant allelic burden in MPN patients. Previous studies in a Jak2 mutant mouse model have shown that IFNα treatment is capable of increasing the proliferation of Jak2 mutant HSCs and depleting them from the bone marrow (175,176). This was also shown to be concomitant with an increased acquisition of DNA damage in the mutant HSCs (176). This suggests a potential mechanism as to how IFNα is able to achieve molecular remission in patients. However, as I have identified the presence of a dormant mutant fraction of HSCs, further questions arose as to whether IFNα is able to specifically target these dormant MPN stem cells and deplete them. Furthermore, comparison of the observed effect of IFNα on mutant HSCs to that of JAK inhibitors has not been explored in previous studies in literature. Hence, as part of my study, I also compared the effects of a JAK

inhibitor (Fedratinib) to that of peg-IFNα, not only in terms of their effects on overall disease phenotype, but importantly the dormant mutant HSC fraction.

### 4.2.1. Both JAK inhibitor and peg-IFNα treatment leads to alleviation of MPN symptoms but only peg-IFNα has an effect on the mutant allelic burden

The Jak2-V617F mutant mouse model used in this study showed elevated peripheral blood hematocrit, splenomegaly and accumulation of immature erythroid cells in the spleen, all of which are classical signs of polycythemia vera (PV). Hence, these three phenotypes in the mice were considered as MPN-associated symptoms, and the effects of Fedratinib and peg-IFNα (lower 300ng and higher 600ng doses) on PV-symptoms were studied. Both Fedratinib and peg-IFNα was observed to have an effect on the aforementioned MPN-symptoms, albeit at slightly varying degrees of efficacy. Fedratinib and peg-IFNα at lower doses was found to prevent further increase in the hematocrit values, suggesting stalled disease progression, but did not significantly reduce the blood hematocrit. This lack of hematocrit reduction has been previously reported in Jak2 mutant mice treated with Fedratinib and is potentially due to the longer half-life of erythrocytes (178,184). However, the 600ng dose of peg-IFNα was found to significantly reduce the hematocrit to normal peripheral blood value. Although IFNα is known to directly reduce erythropoiesis by affecting erythroid progenitors and inhibiting erythropoietin-mediated proliferation (190,191), it was surprising to see such a strong effect in reducing mature erythrocyte count, especially considering their long half-life. This suggests that there might be a potential secondary effect of peg-IFNα on mature erythrocytes that is not yet known. Looking at the phenotype of splenomegaly, both Fedratinib and peg-IFNα was found to be capable of reducing the spleen weight and concomitantly reducing the proportions of accumulated immature erythrocytes in the spleen. Here again, the peg-IFNα at the 600ng dose was capable of reducing the spleen weight the most, compared to Fedratinib and the 300ng dose of peg-IFNα. The treatment duration for Fedratinib in my study was 4 weeks while previous studies involved at least 6 weeks of treatment (178). This could be the reason for the lower efficacy in reducing splenomegaly observed by me. Longer treatment duration might be as efficacious as peg-IFNα. Nevertheless, both therapeutic agents were capable of reducing the symptoms associated with MPN.

JAK inhibitors are known to be capable of reducing disease associated symptoms in human patients of MPN (155,156). However, their main problem lies in their inability to reduce mutant allelic burden in these patients. This is also what I observed in my study with the Jak2 mutant mice. Fedratinib treated mutant mice continued to show increase in the proportion of mutant cells in the peripheral blood during the treatment period. This indicates that Fedratinib has no effect on the mutant allelic burden, and in fact leads to an increase. This could suggest a potential reason as to why JAK inhibitor treatments in human patients of MPN have been associated with primary therapy resistance, sub-optimal response and also cases of post-treatment disease relapse (192–194). In contrast to Fedratinib treatment, peg-IFNα treated mutant mice did not show an increase in the percentage of mutant cells in the peripheral blood, indicating a stabilization of the mutant allelic burden and deterrence of disease progression. The 600ng dosage of peg-IFNα was even capable of reducing the proportion of peripheral blood mutant cells in some of the mice. This highlights the better efficacy of peg-IFNα over Fedratinib, in reducing the mutant allelic burden in MPNs, as has been reported in human patients receiving IFNα therapy (174).

### 4.2.2. Only IFNα treatment can deplete the dormant fraction of MPN stem cells indicating its mechanism of action in achieving molecular remission

Having validated that the differential effects of Fedratinib and peg-IFNα on the mutant allelic burden is similar to that observed in humans, I sought to understand the reason behind this observation. Since the Jak2 mutant HSCs are known to act as the disease initiating and propagating MPN-stem cells (135), I first analyzed the frequency of mutant HSCs following treatment with Fedratinib or the two doses of peg-IFNα. It was observed that the frequency of mutant HSCs was unaltered in the bone marrow of mutant mice treated with Fedratinib. This is in line with previous observations in Jak2 mutant mouse model by Mullally *et al* (178). Mutant mice treated with the high dose peg-IFNα on the other hand were found to have a significant reduction in the frequency of mutant HSCs in the bone marrow. This is again consistent with other studies of Jak2 mutant mice, where IFNα was capable of depleting the mutant HSCs in the bone marrow (175,176). The 300ng dose of peg-IFNα was not capable of reducing the number of mutant HSCs. However, this low dose was

able to stop the increase in mutant allelic burden in the peripheral blood, similar to the high dose peg-IFNα. This suggests that the lower 300ng dosage still has an effect on the mutant HSCs in their function of disease propagation. This dose might hence need a longer duration of observation to see an effective depletion of the mutant HSCs from the bone marrow.

The existence of the dormant fraction of the Jak2 mutant HSCs in the bone marrow that I identified, and previous studies indicating the deleterious effects of IFNα-mediated proliferation on the functional potency of wildtype HSCs, led me to hypothesize that peg-IFNα might be depleting the mutant HSCs by pushing them out of dormancy. To study this, I performed the BrdU pulse-chase experiment while treating the Jak2 mutant mice with either Fedratinib or the two doses of peg-IFNα. Fedratinib treatment did not lead to any decrease in the percentage of dormant mutant HSCs in the bone marrow. On the other hand, both doses of peg-IFNα was observed to decrease the proportion of dormant mutant HSCs. This in in conjunction with the observation that peg-IFNα treatment reduces the frequency of mutant HSCs in the bone marrow and also prevents increase in peripheral blood mutant allelic burden.

Together, the observations made in my study points to the depletion of the dormant fraction of Jak2 mutant HSCs, as the potential mechanism of action of IFNα in achieving molecular remission in patients.

## 4.3. Combination treatment of peg-IFNα with other drugs could be used to reduce IFNα dosage and thereby side effects

One of the major limitations of IFNα as a therapeutic option in treating MPNs is that patients drop out of treatment due to adverse side effects, even though the efficacy of the treatment in achieving molecular remission is very high (173). This makes the potential mechanism of action of IFNα, identified in this thesis, an important means to further the therapeutic options available for patients. For example, a combination treatment of peg-IFNα with Fedratinib could be evaluated. The lower dose of peg-IFNα, even though efficient in depleting dormant mutant HSCs, was less efficient in treating the disease symptoms compared to the higher dose. Lowering the dose could be a way to reduce the occurrence or degree of adverse effects that are associated with IFNα treatment. Hence, by combining the Fedratinib and low

dose peg-IFNα administration, the dormant mutant HSCs could still be targeted along with a theoretical increase in efficacy of treating overall disease symptoms. Combinatorial treatments of IFNα and another JAK inhibitor Ruxolitinib has been studied recently in clinical trials and found to be effective in reducing mutation burden in PV and myelofibrosis patients (195). However, Ruxolitinib is known to inhibit both JAK1 and JAK2 (196,197) and IFNα signalling cascade involves JAK1 signal transduction (198). Hence the efficacy of IFNα might be reduced by combining it with Ruxolitinib treatment, and inhibitor such as Fedratinib which is more specific to JAK2, might be better. Peg-IFNα treatment of Jak2 mutant mice has also been shown to specifically induce more DNA damage in the mutant HSCs compared to normal HSCs (176). This would suggest that combinatorial treatment with drugs that target DNA damage response, such as PARP inhibitors, might be able to sensitize the mutant HSCs to IFNα induced DNA damage, thereby increasing the efficacy of IFNα in depleting the mutant HSCs. Treatments involving arsenic trioxide along with IFNα has also been shown to be efficient in treating MPNs in a Jak2V617F mouse model (199). The authors studied the role of PML nuclear bodies as the reason for the efficacy of treatment, but arsenic trioxide is also known to induce DNA damage in dividing cells (200). Hence, their observations add further value to the theory of combinatorial treatments of peg-IFNα with drugs that induce DNA damage or target DNA damage response.

In conclusion, the observation that lowering the dose of peg-IFNα is still able to deplete the dormant fraction of Jak2 mutant HSCs in the bone marrow, opens the door for testing potential combinatorial treatments of low dose peg-IFNα with other drugs.

## 4.4. Limitations of the study

### 4.4.1. Limitations to the use of BrdU pulse-chase assay as a method to study HSC dormancy

BrdU pulse-chase experiments were performed to assess mutant HSC dormancy in this chapter of thesis. For this, the mice were administered with BrdU through drinking water for a period of 14 days to pulse-label the DNA in the HSCs with BrdU incorporation. Stopping the administration of BrdU starts the chase period,

and the proliferating cells start to dilute the amount of BrdU in the cells, as the daughter cells will only have half the initial amount of BrdU after each division. The dormant cells during the period of chase would retain the incorporated BrdU in the DNA. This was used as a measure of HSC dormancy. However, BrdU incorporation into the DNA is itself dependent on DNA replication during cell proliferation. Hence, the deeply dormant HSCs during the labelling period will not incorporate the BrdU in their DNA. This was evident from the observation that only 70-80% of HSCs are initially labelled with BrdU before the start of the chase period. The remaining 20% unlabelled mutant HSCs that did not incorporate BrdU could be more deeply dormant. The actual percentage of dormant Jak2 mutant HSC fraction in the bone marrow of Jak2 mutant mice, might hence be even higher than what is observed here, thereby undermining the proportions. Also, while we observed the elimination of the label-retaining dormant fraction of mutant HSCs post-treatment with both doses of peg-IFNα, it is not possible to know whether the unlabelled dormant mutant HSCs were also eliminated by the treatment. A longer administration of BrdU -pulse to label more HSCs is also not an option, as BrdU treatment more than 14 days is toxic to mice. Hence, an initial labelling mechanism which is independent of proliferation would be needed here to get a more accurate picture of the true proportion of dormant disease propagating HSCs.

### 4.4.2. Unknown proportion of dormant HSCs that have achieved Cre-induced recombination of the JAK2V617F allele

The efficiency of Cre recombinase in terms of the percentage of cells that had a successful recombination of the transgene can vary depending on the promoter under which the Cre is expressed in the mouse (201). Previous studies using the JAK2V617F mouse model, that was used as part of this thesis, expressed Cre under the control of E2A or Vav promoters (178). However, in the work presented in this thesis, the Cre was under the Rosa26 promoter. Hence, the recombination efficiencies could be different to that previously known, and might be less than 100%. If this is the case, then it is not possible to know if the label-retaining dormant mutant HSCs are in fact genetically recombined and express the mutant Jak2. They could be non-recombined cells and could be essentially wildtype cells, which would cast doubt on the existence of dormant mutant HSCs. It is hence important to isolate the dormant HSCs from the Jak2 mutant mice and assess the frequency of recombined cells within this fraction.

However, since the BrdU staining involves a DNase digestion step, it is not possible to genotype the isolated dormant cells. Therefore, a labelling system which can be used to isolate dormant cells without fixation or DNase treatment is required to assess this potential limitation.

## 4.5. Conclusions and future perspective

The primary aim of this project was to understand the yet unknown mechanism of action of IFNα in being the only drug capable of achieving molecular remission in MPN patients, in comparison to JAK inhibitor therapy. Firstly, I identified that a small fraction of Jak2V617F mutant HSCs in the bone marrow of mice with polycythemia vera, existed in a dormant state, even though a majority of the mutant HSCs exhibited increased proliferation. These dormant mutant HSCs could be potentially driving the disease and were hypothesized to be offering resistance to JAK inhibitor therapy in terms of reducing the mutant allelic burden. This was indeed observed to be the case, wherein Fedratinib (JAK2 inhibitor) was unable to elicit an effect on the dormant mutant HSC fraction and was unable to reduce the frequency of mutant HSCs in the bone marrow. This was concomitant with a lack of effect on the peripheral blood mutant allelic burden. The observation hence suggests that the lack of mutant burden response to JAK inhibitor therapy in patients, could be due to a lack of elimination of the dormant mutant HSCs. In contrast to Fedratinib, peg-IFNα treatment of mutant mice was found to drive the dormant mutant HSCs into proliferation and significantly reduce the frequency of mutant HSCs in the bone marrow, along with a reduction in MPN-associated symptoms similar to Fedratinib. The depletion of dormant mutant HSCs by peg-IFNα also correlated with a prevention of increase in the mutant burden in the peripheral blood. This concludes that only peg-IFNα is capable of mediating an effect on the Jak2 mutant HSCs in MPNs. Hence, the push of dormant mutant HSCs into proliferation and their subsequent depletion from the bone marrow, is the potential mechanism of how IFNα therapy is capable of achieving molecular remission in MPN patients.

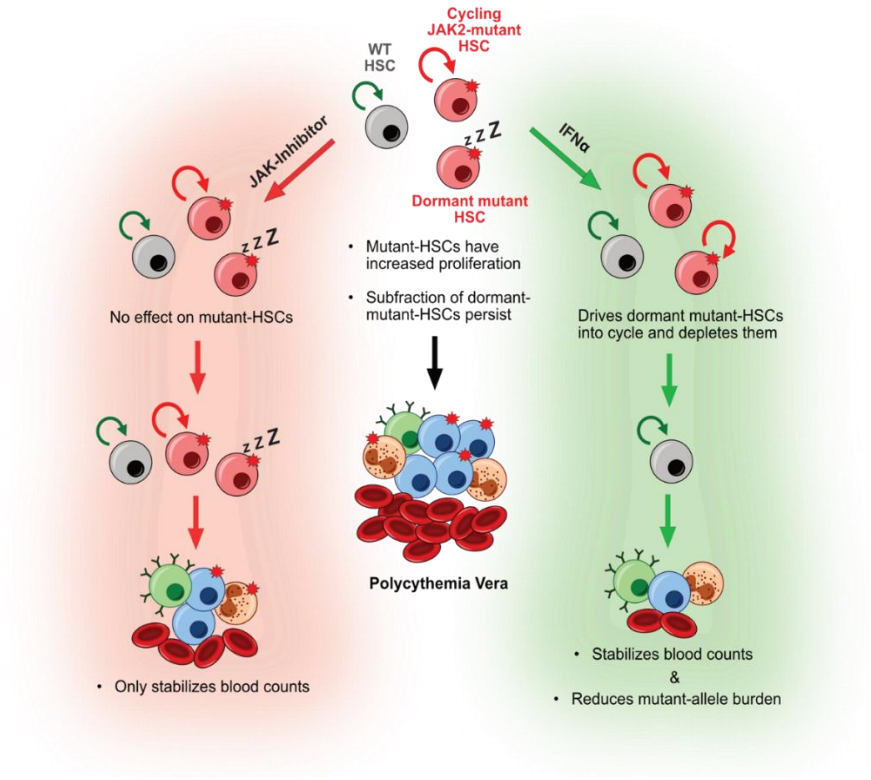


**Figure 4.1: Graphical summary of the study to elucidate the mechanism of action of peg-IFNα in the treatment of myeloproliferative neoplasms**

The observations of elimination of dormant mutant HSCs and the effect on prevention of increase in mutant burden in mice treated with peg-IFNα, is only a correlative assessment in this study. This brings forth two important questions to be answered to further validate this mechanism of action of peg-IFNα. Firstly, it is unclear whether the dormant and the proliferating fractions of the mutant HSCs, differ in their potential to initiate and propagate disease. Furthermore, the question of whether driving the dormant mutant HSCs into proliferation by administering peg-IFNα leads to a definitive reduction in the frequency of disease initiating cells. To answer these questions, a limiting dilution transplantation assay of the dormant and proliferating Jak2 mutant HSC fractions have to be performed, pre- and post-treatment with peg-IFNα. However, BrdU labelling is not suitable here, since it is not possible to sort live BrdU-positive cells, as the staining methodology involves formaldehyde fixation of the cells. For this purpose, a transgenic H2B-GFP mouse model that has been previously used to assess HSC dormancy in wildtype mice (25), can be used. This transgenic mouse model would allow us to perform the pulse-chase

experiment followed by the isolation of live dormant and proliferating HSCs. Combining this model with the Jak2 mutant mouse model is currently being evaluated. With the knowledge that peg-IFNα induces more DNA damage in the mutant HSCs compared to wildtype HSCs, further follow up studies to identify potential molecular pathways involved in this DNA damage response is also needed. This would enable identification of potential combinatorial treatments of peg-IFNα along with a drug that targets DNA damage response, and sensitize the HSCs to peg-IFNα treatment. This could increase the therapeutic window of peg-IFNα, allowing for a lower dose to be used, and subsequently mitigate treatment associated side effects, which is one of the most important limitations of IFNα therapy.

# 5 Results-II

## Co-housing model to assess the effects of wild microbiota infections on hematopoiesis

Multiple mouse models have been previously used to study infection-induced changes to the hematopoietic system and specifically the biology of HSCs in response to infection-stress. These include injecting mice with pI:pC (viral dsRNA mimetic) or directly injecting mice with pathogenic microorganisms such as Mycobacterium avium and murine cytomegalovirus (mCMV) (28,30,76). However, such models have potential limitations relating to their biological relevance. For example, the dosage and scheduling of pI:pC treatment may not recapitulate the inflammatory response generated during an actual viral infection, while injecting purified pathogens into mice is usually performed at a non-physiological high dose of infectious units administered via route that does not mimic the natural infection process. In this thesis, I have assessed the hematologic effects of a novel co-housing model system, which allows natural transmission and colonization of wild microbiota into clean laboratory mice that are normally housed under specific pathogen free (SPF) hygiene conditions.

## 5.1. Co-housing clean laboratory mice with wildling mice leads to effective transfer and colonization of laboratory mice with wild microbiota

The infectious mice used in all experiments in this part of the thesis are referred to as “wildling” mice. These mice are C57BL6/NTac mice and carry a range of wild mouse microbiota **(Figure 5.1).** The wildling mice were originally generated and maintained by the lab of Dr. Stephan Rosshart and were provided for the experiments by them. Six of the wildling mice used in the experiments were subject to microbiology

analysis. Samples such as blood serum, fur swab, oral swab and sections from various organs were collected. Serology analysis was performed to identify pathogen associated serum antibodies, as a retrospective analysis of previous infections, while cultures and PCRs for microorganisms in different organs were performed to identify active pathogens in the mice. These microbiological tests were performed by the Microbiology facility at the DKFZ under Dr. Katja Schmidt. Commensals such as murine Noroviruses, Astroviruses, *Rodentibacter* and *Muribacter* were present in all wildling mice. Helicobacter infections were also observed to be common across all wilding mice tested. All tested mice were also found to have mites on their fur as exoparasites. The strains of *Rodentibacter* and *Muribacter* identified were however different among the mice. Additionally *Haemophilus influenzae murium* infections were found in one-third of the wildling mice. All in all, the wildling mice tested here showed a small heterogeneity in the presence of different infectious microorganisms.

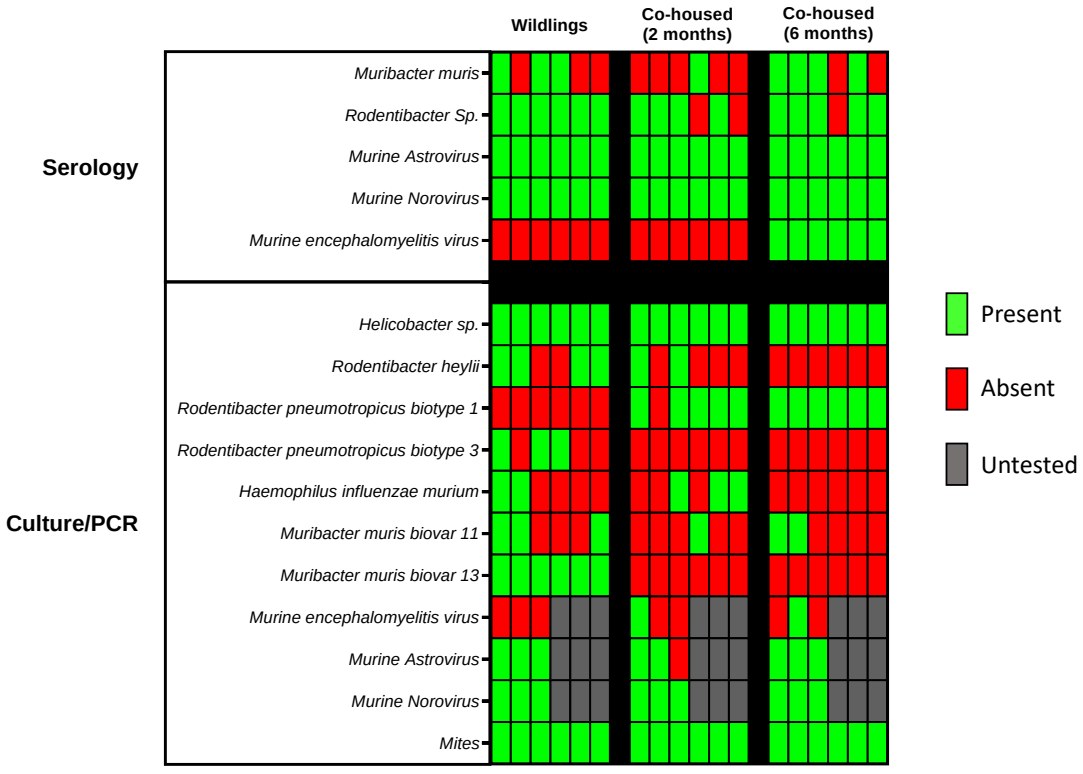


**Figure 5.1: Co-housing enables horizontal transfer and colonization of wild microbiota from wildling mice to clean laboratory mice**

The wildling mice were used to perform co-housing experiments to allow the natural transmission and colonization of the different wild microbes into "clean" laboratory mice, previously bred and housed under SPF hygiene conditions. Wildtype SPF C57BL/6J mice were co-housed with a wildling mouse in the same cage for a period of 3 weeks and then the wildling mouse was removed from the cage. The wildling mouse in each co-housing cage was changed once per week, so that the SPF mice in each cage encountered different wildling mice to account for the heterogeneity of the microbiota spectrum in the wildling mice. The co-housed mice were then also subject to the same microbiological tests as the wildling mice at 2 months and 6 months from the start of co-housing. The 2 month time-point would reflect an acute state of infection and the 6 month's time-point reflects a long-term infection and colonization of the wild microbiota.

I observed successful horizontal transmission of various infectious agents from the wildling mice to the co-housed mice. This was evident through both the serology analysis and the bacterial culture and PCR analysis **(Figure 5.1)**. Co-housed mice analyzed at both the 2 months and 6 months time-points had antibodies against the different commensal organisms in the wildling mice, suggesting that the co-housed mice had a humoral immune response against them. The presence of active infections such as Helicobacter and the other commensals show an active colonization of these organism for at least up to 6 months of experimental duration. A certain proportion of the co-housed mice showed active infections by the murine encephalomyelitis virus and the 6 months co-housed mice also had an antibody response against this pathogen. However, this was not present in the wildling mice that were tested, suggesting some of the untested wildling mice that were used for the co-housing might have had this infection. This again points to some level of heterogeneity of pathogen spectrum across the different wildling mice and also the co-housed mice analyzed.

## 5.2. Assessing the acute effects of co-housing mediated horizontal transfer of wild microbiota to clean laboratory mice

With the effective transmission of infections during co-housing, I wanted to assess the acute effects of infections on hematopoiesis. To this end, the co-housed mice were assessed for peripheral blood counts, bone marrow hematopoietic cells and

hematopoietic stem cell fitness after acute infections. Wildtype C57BL/6J mice housed under SPF hygiene conditions were used as clean control mice for all analysis.

### 5.2.1. Co-housing mediated transfer of wild microbiota leads to elevated peripheral blood cell counts 2 months from start of co-housing

After 2 months from the start of co-housing, blood samples were collected from the co-housed mice and the blood was analyzed using a blood cell counter. The co-housed mice showed significantly elevated overall peripheral blood white cell, lymphocyte, monocyte and granulocyte counts **(Figure 5.2A-D)**. The increased number of peripheral blood monocytes and granulocytes suggests an active innate immune response against the transmitted infections. The elevated levels of lymphocytes suggest that the mice also have an active adaptive immune response against the respective pathogens. This is also evident from the presence of antibodies against the infections in the serology tests **(Figure 5.1)**. However, there were no changes in the erythrocyte and platelet counts in the co-housed mice compared to the clean control mice.

Since there were global changes in the peripheral blood cell counts in the co-housed mice, I further accessed the different subsets of myeloid and lymphoid cells in the peripheral blood using flow cytometry **(Figure 5.3)**. Overall, myeloid cell counts in the peripheral blood along with different monocyte subsets were assessed. The CD11b+ myeloid cells were further analyzed for the two different monocyte subsets based on their surface Ly6C expression levels. The presence of these monocyte subsets can act as an indicator of the innate immune response to infections. Additionally the proportions of the B-lymphocytes and the different T-lymphocyte subsets that respond to infections were also studied. CD4 T-helper cells and the CD8 cytotoxic T-cells were sub-categorized into their naïve, active and memory stages using CD62L and CD44 surface marker expression levels. This gave an insight into the level of adaptive immune response happening in the mice at this acute phase of infection.

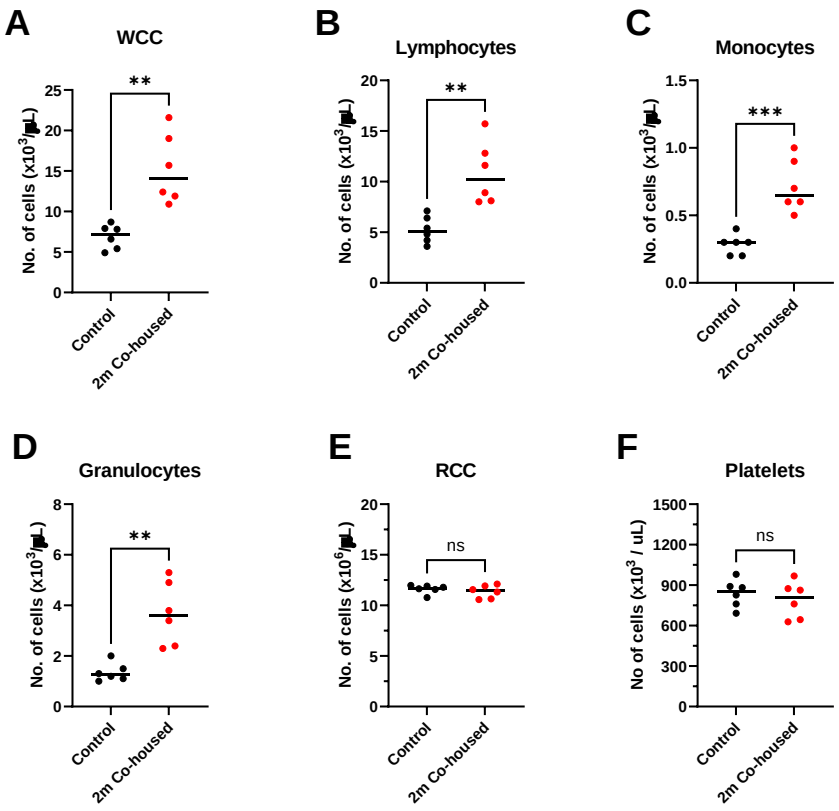


**Figure 5.2: Co-housed mice show elevated peripheral blood counts 2 months from the start of co-housing**

**(A)** Peripheral blood white cell count (WCC) at 2 months after co-housing. **(B)** Peripheral blood lymphocyte count at 2 months after co-housing. **(C)** Peripheral blood monocyte count at 2 months of co-housing. **(D)** Peripheral blood granulocyte counts at 2 months of co-housing. **(E)** Peripheral blood red cell count (RCC) at 2 months of co-housing. **(F)** Peripheral blood platelet count at 2 months of co-housing.

*(n=6 per group; All values are mean±SD; ** represents P<0.01, *** represents P<0.001 and ns represents non-significant)*

In line with the observed increase in granulocytes and monocytes in the peripheral blood, as assessed using a blood cell counter, the flow cytometry analysis also showed significantly elevated number of myeloid cells in the peripheral blood of the co-housed mice **(Figure 5.4A)**. Both the Ly6C$^{High}$ and Ly6C$^{Low}$ monocytes were observed to be significantly higher in number in the co-housed mice compared to the clean control mice. Ly6C$^{Low}$ monocytes are referred to as "patrolling" monocytes and are usually early responders to inflammation (202). Ly6C$^{High}$ monocytes on the other hand are pro-inflammatory/anti-microbial monocytes that can carry antigens and differentiate into macrophages or dendritic cells depending on the cytokines present at the site of infection or inflammation (203,204). The increased presence of these

monocytes suggests a high acute innate immune response against the transmitted infections and the accompanied inflammatory response during the co-housing phase. The co-housed mice were also observed to have an increased number of B-lymphocytes, CD4+ T-helper cells and CD8+ cytotoxic T-cells in the peripheral blood **(Figure 5.4D-F)**, indicative of an active adaptive immune response.

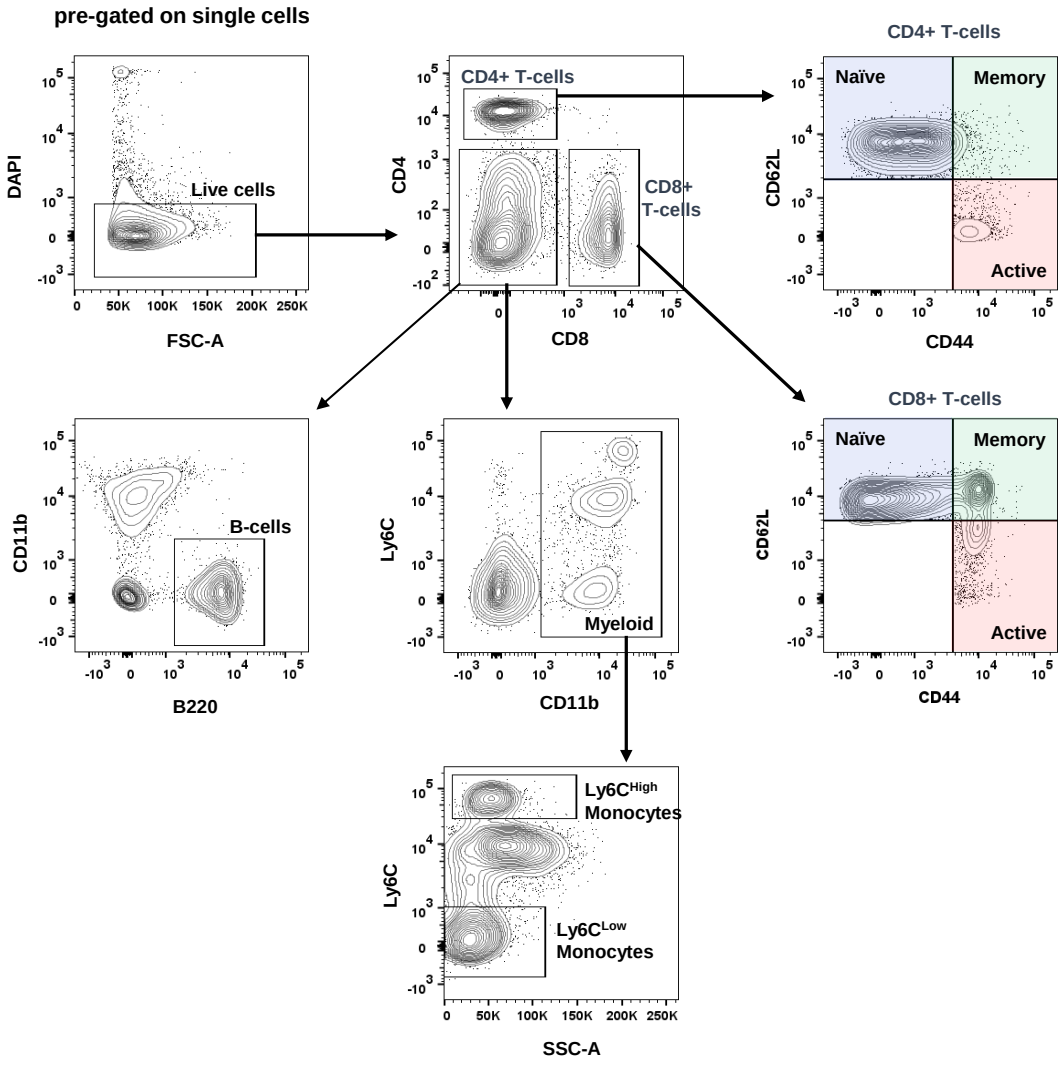


Figure 5.3: Flow cytometry scheme to analyze different mature cell subsets in the peripheral blood

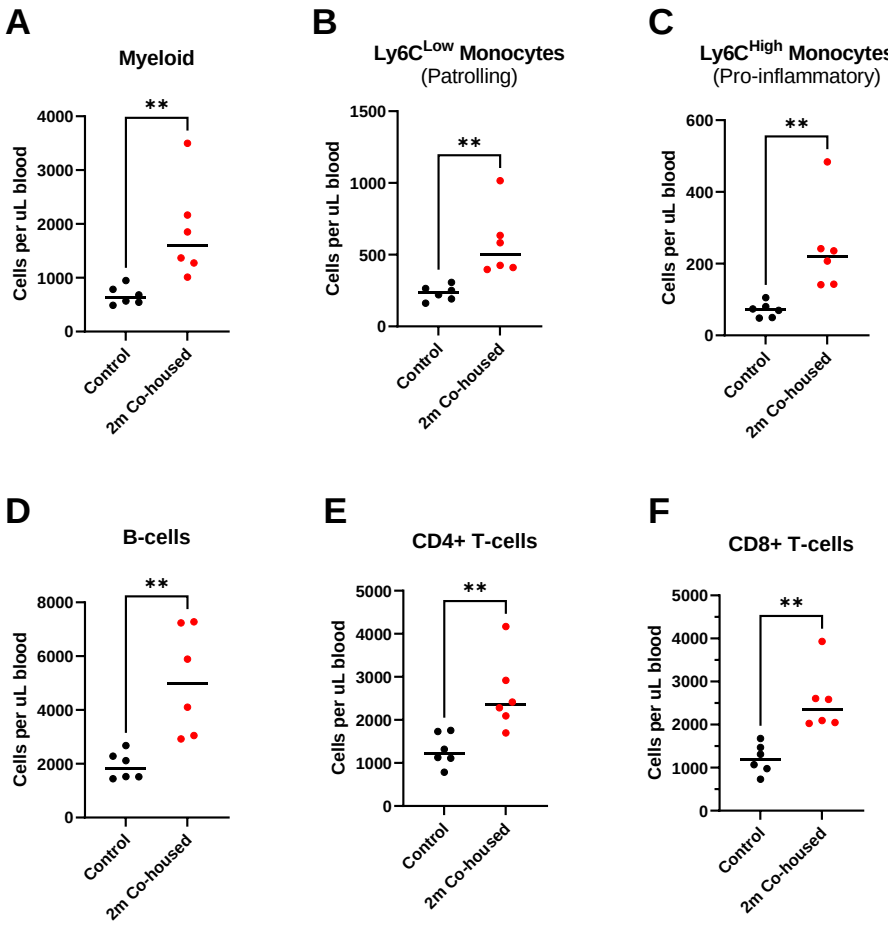


**Figure 5.4: Co-housed mice show elevated peripheral blood mature cells at 2 months from start of co-housing**

**(A)** Peripheral blood myeloid cell count at 2 months after co-housing. **(B)** Peripheral blood patrolling monocyte count at 2 months after co-housing. **(C)** Peripheral blood pro-inflammatory monocyte count at 2 months of co-housing. **(D)** Peripheral blood B-cell counts at 2 months of co-housing. **(E)** Peripheral blood CD4+ T-cell count at 2 months of co-housing. **(F)** Peripheral blood CD8+ T-cell count at 2 months of co-housing.

*(n=6 per group; All values are mean±SD; ** represents P<0.01 and ns represents non-significant)*

I also observed that the co-housed mice had elevated proportions of CD8+ memory T-cells **(Figure 5.5C-D)**. Although there was no statistically significant difference in the proportions of active CD8+ T-cells in the co-housed mice compared to the clean control mice, there was an increasing trend observed in some mice as can be seen in the representative flow cytometry plot **(Figure 5.5C)**. The active CD8+ T-cells have been previously shown to reduce in number post-infection response with a concomitant increase in memory cells (205,206). This would suggest that in some mice the acute phase of infection might already be close to ending. CD4+ T-cell activation is

also known to be usually slower than CD8+ T-cell activation (205), which could explain the increased proportions of CD4+ T-cells at the 2 month time-point.

In conclusion, the co-housed mice show an overall increase in mature cell composition in the peripheral blood after 2 months of co-housing, indicating an acute response to the spectrum of wild microbiota transferred from the wildling mice.

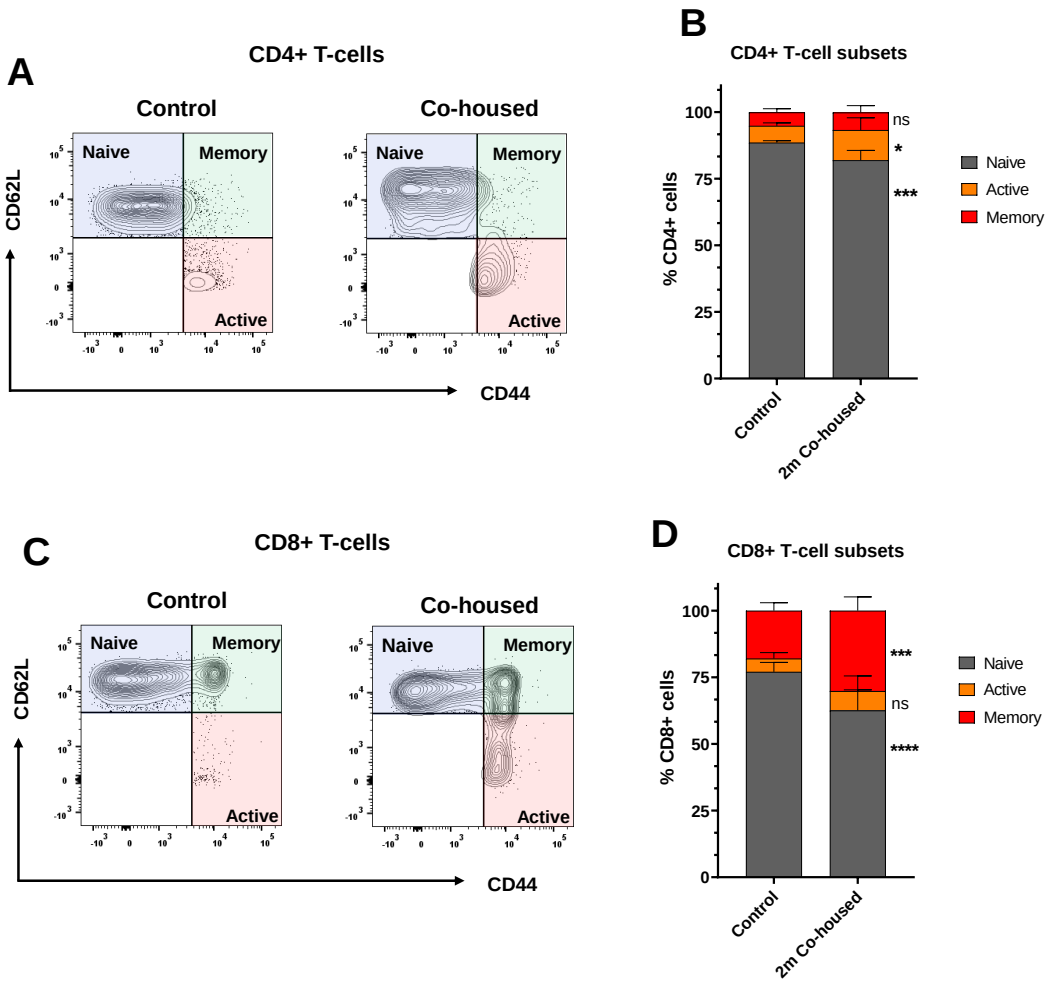


**Figure 5.5: Co-housed mice show increased proportion of active and memory T-cells 2 months from the start of co-housing**

**(A)** Representative flow cytometry plot representing the different CD4+ T-cell subsets. **(B)** Proportions of the different CD4+ T-cell subsets in the peripheral blood of 2 months co-housed and control mice. **(C)** Representative flow cytometry plot representing the different CD8+ T-cell subsets. **(D)** Proportions of the different CD4+ T-cell subsets in the peripheral blood of 2 months co-housed and control mice.

*(n=6 per group; All values are mean±SD; * represents P<0.05, *** represents P<0.001, **** represents P<0.0001 and ns represents non-significant)*

### 5.2.2. Co-housed mice do not show any changes in the number of oligopotent progenitor cells due to the acute infection response

Since I observed changes in the peripheral blood mature cells in the co-housed mice, I then assessed the number of oligopotent progenitor cells in the bone marrow to understand if the increased mature cells production results in a depletion or expansion of the oligopotent progenitors in the bone marrow. The number of oligopotent progenitor cells, referred to as LK (Lineage$^-$, cKit$^+$, Sca1$^-$) cells, was analyzed by flow cytometry of the bone marrow cells **(Figure 5.6)**. The oligopotent progenitor compartment consist of the Megakaryocyte-Erythrocyte Progenitor (MEP), Common Myeloid Progenitor (CMP), Granulocyte-Monocyte Progenitor (GMP) and Common Lymphoid Progenitor (CLP). The CMPs were further sub-divided into CD55+ CMPs which are known to be primed towards megakaryocytic and erythroid differentiation (207).

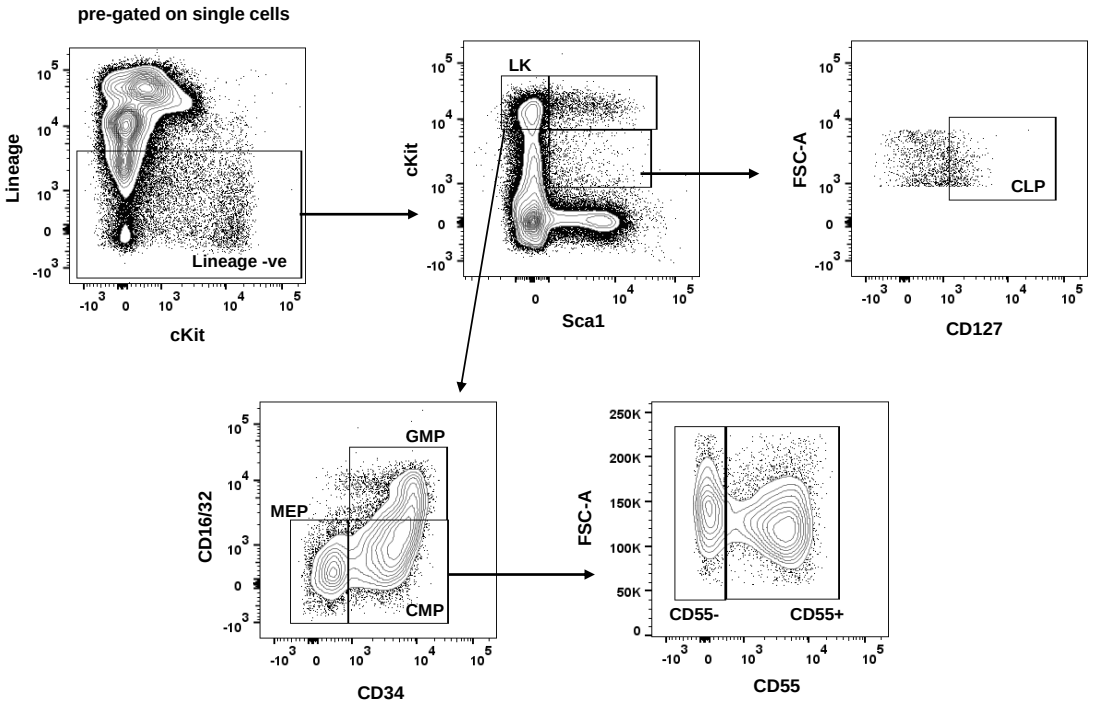


Figure 5.6: Flow cytometry scheme to analyze oligopotent progenitor cells in the bone marrow

There was no difference observed in the absolute number of CMPs, GMPs, MEPs or CLPs in the bone marrow of co-housed mice compared to clean control mice **(Figure 5.7A-D)**. The lack of change in the number of CMPs and GMPs is intriguing considering the apparent increase in mature granulocytes and monocytes in the peripheral blood of the co-housed mice. Looking at the number of CD55+ CMPs, there was again no changes observed in the co-housed mice **(Figure 5.7E)**. However, this is as expected since there was no change observed in the number of erythrocytes or platelets in the peripheral blood. Furthermore, there was also no difference observed in the proportion of CMPs, MEPs and GMPs within the LK compartment in the bone marrow of co-housed mice **(Figure 5.7F)**, suggesting that there is no bias towards any specific differentiation trajectories during the acute infections phase in the co-housed mice at the 2 month's time-point. This could be due to the demand for mature peripheral blood cells being met by the differentiation of more lineage restricted progenitors downstream of the oligopotent progenitor cells during the acute phase of the infection response after co-housing.

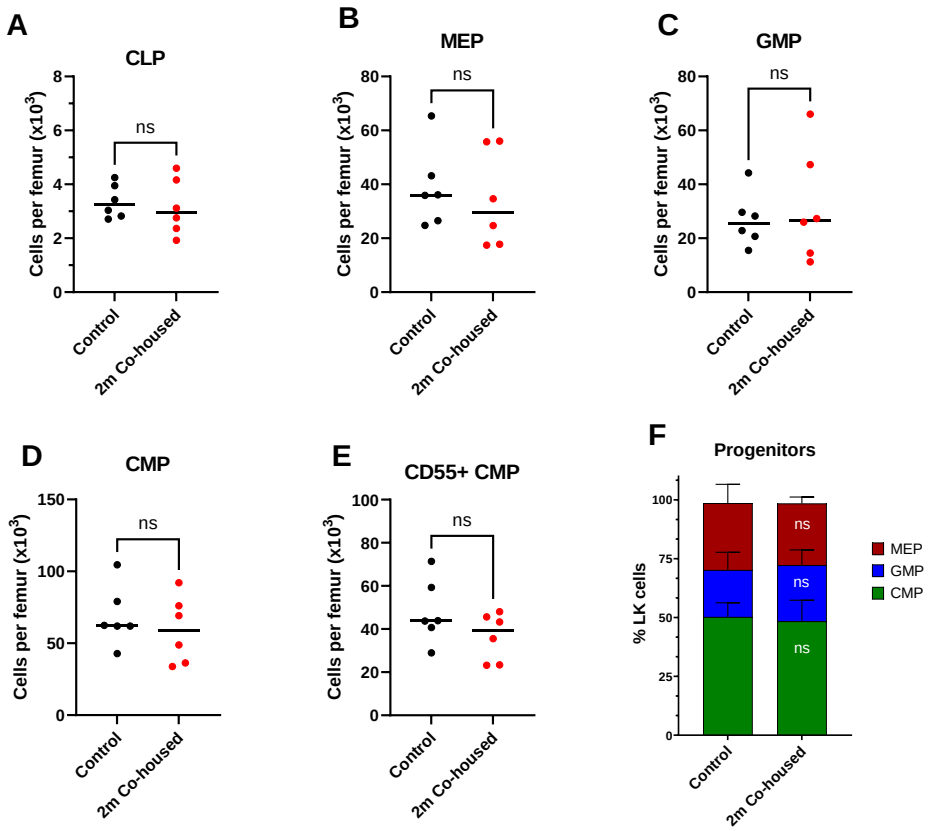


Figure 5.7: Co-housed mice do not exhibit any changes in the numbers of different types of oligopotent progenitor cells in the bone marrow

**(A)** Number of common lymphoid progenitor (CLP) cells in the bone marrow at 2 months after co-housing. **(B)** Number of megakaryocyte-erythrocyte progenitor (MEP) cells in the bone marrow at 2 months after co-housing. **(C)** Number of granulocyte-monocyte progenitor (GMP) cells in the bone marrow at 2 months after co-housing. **(D)** Number of common myeloid progenitor (CMP) cells in the bone marrow of 2 months co-housed mice. **(E)** Number of CD55+ CMPs in the bone marrow at 2 months after co-housing. **(F)** Proportion of MEPs, CMPs, and GMPs within the LK cell compartment in the bone marrow at 2 months after co-housing.
*(n=6 per group; All values are mean±SD; ns represents non-significant)*

### 5.2.3. Co-housed mice do not show any changes in the number of HSPCs in the bone marrow due to the acute infection response

Having analyzed the oligopotent progenitor cells in the bone marrow, I next assessed whether there were any changes in the hematopoietic stem and progenitor cell (HSPC) compartments, which are the most primitive hematopoietic cells in the bone marrow. Flow cytometry was used to analyze the different cell types within the HSPC cellular compartment **(Figure 5.8)**. The overall HSPC compartment is referred to as LSK (Lineage$^-$ Sca1$^+$ cKit$^+$). The multipotent progenitor cells were further sub-categorized as MPP2, MPP3, MPP4 and MPP5. MPP1 cells are referred to as short-term repopulating HSCs (ST-HSCs) and are hence included in the HSC compartment.

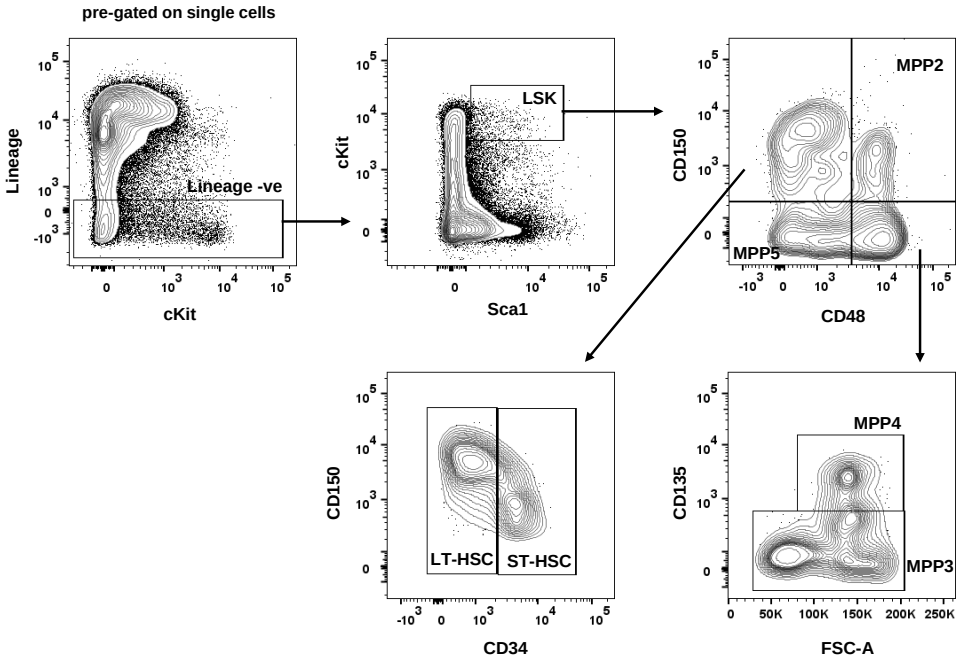


**Figure 5.8: Flow cytometry gating scheme to analyze the hematopoietic stem and progenitor cells (HSPCs)**

There was no significant changes observed in the number of the different MPP subtypes in the bone marrow of 2 months co-housed mice compared to the clean control mice **(Figure 5.9A-D)**. There was a small trend towards increased number of MPP3 cells **(Figure 5.9B)**, which are known to be primed towards myeloid differentiation trajectory (208). The elevated number of myeloid cells observed in the peripheral blood of the co-housed mice might be driving the increase in demand for myeloid differentiation leading to increased MPP3 proliferation in the bone marrow. However, since the change is not statistically significant, further replicates are required to confirm the observation. There were also no differences observed in the number of short-term HSCs (ST-HSCs) or long-term HSCs (LT-HSCs) in the bone marrow of co-housed mice **(Figure 5.9E-F)**. This suggests that under a more natural transmission of mild infections, the HSC compartment might not be required to engage directly in driving the hematopoietic demand at least during acute infections.

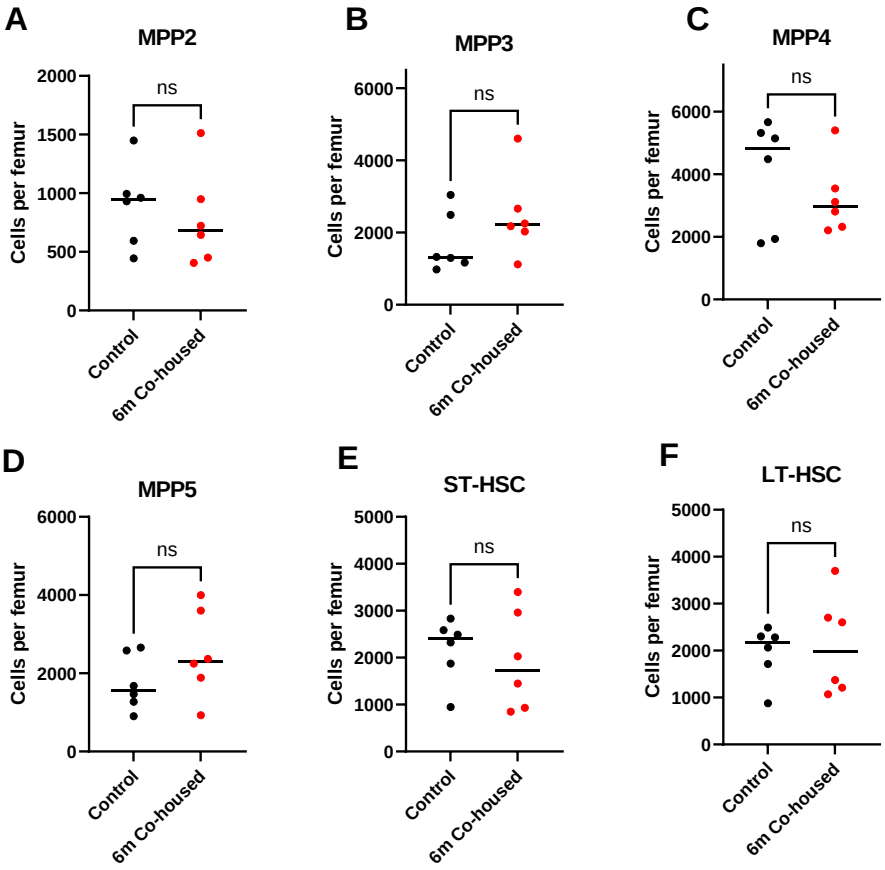


**Figure 5.9: Co-housed mice do not exhibit any changes in the numbers of hematopoietic stem and progenitor cells (HSPCs) in the bone marrow**

**(A)** Number of MPP2 cells in the bone marrow at 2 months after co-housing. **(B)** Number of MPP3 cells in the bone marrow at 2 months after co-housing. **(C)** Number of MPP4 cells in the

bone marrow at 2 months after co-housing. **(D)** Number of MPP5 cells in the bone marrow of 2 months co-housed mice. **(E)** Number of ST-HSCs in the bone marrow at 2 months after co-housing. **(F)** Number of LT-HSCs in the bone marrow at 2 months after co-housing.
*(n=6 per group; All values are mean±SD; ns represents non-significant)*

### 5.2.4. Competitive transplantation assay shows that acute infections from wild microbiota does not impair HSC engraftment potential

Even though there were no changes observed in the number of HSCs in the bone marrow of co-housed mice at the acute 2 month time-point, I wanted to assess the fitness of those HSCs in repopulating the hematopoietic system. To this end, I performed a competitive bone marrow transplantation assay **(Figure 5.10A)**. Bone marrow from 2 months co-housed mice (CD45.1) was mixed with bone marrow from wildtype competitor mouse (CD45.1/2) in 1:1 ratio, and transplanted into lethally irradiated recipient mice (CD45.2). The recipient mice were then bled once every 4 weeks to assess the percentage of CD45.1 cells in the peripheral blood. Recipient mice transplanted with bone marrow from age-matched SPF CD45.1 mice were used as control. The recipient mice were euthanized 24 weeks post-transplant to also access the percentage of HSPC engraftment in the bone marrow.

There was no significant difference observed, between mice transplanted with co-housed or control bone marrow, in the percentage of CD45.1 cells in the peripheral blood from 4 to 24 weeks post-transplant **(Figure 5.10B)**. Hence, there were not defects in the initial engraftment of HSCs from co-housed mice and also no long-term repopulating defects in the HSCs. This would suggest that acute effects of co-housing mediated infections does not alter HSC fitness as assessed by competitive transplantation. However, analysis of the bone marrow of mice transplanted with co-housed mouse bone marrow showed a trend towards reduced percentage of overall LSK cells (HSPCs), with a particular reduction in the LT-HSCs in these mice **(Figure 5.10C-E)**. This would suggest a long-term engraftment defect of HSCs from some co-housed mice due to the acute effects of infections. However, this difference was not statistically significant and requires further experimental repeats.

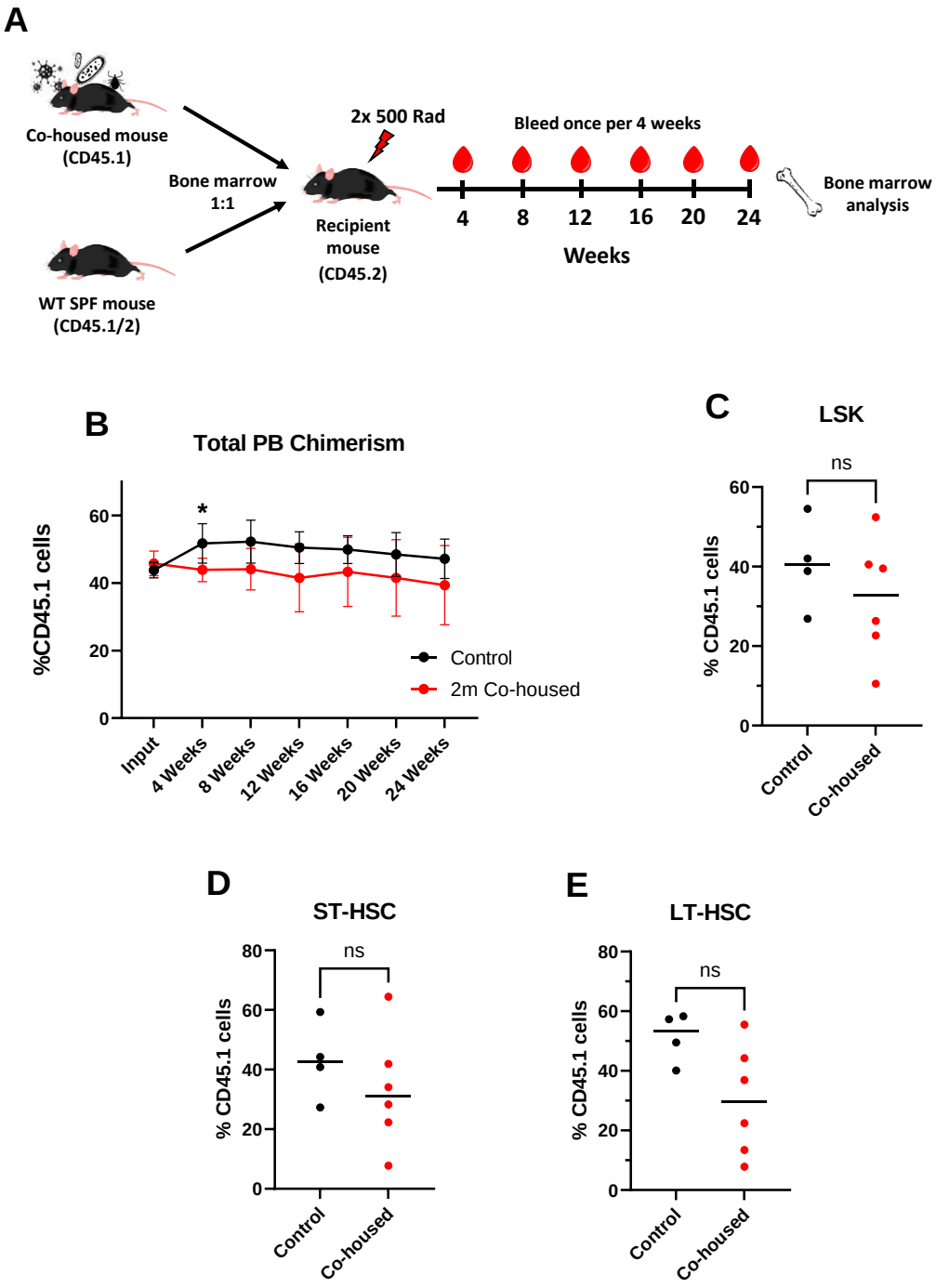


**Figure 5.10: Competitive transplantation assay shows that acute infections from wild microbiota does not impair HSC engraftment potential**

**(A)** Experimental scheme for competitive transplantation assay to assess HSC functional potency. **(B)** Change in total peripheral blood donor (CD45.1 co-housed or control mice) chimerism over 24 weeks from transplantation **(C)** Percentage of CD45.1 donor LSK cells in the bone marrow of competitively transplanted mice at 24 weeks post-transplant. **(D)** Percentage of CD45.1 donor ST-HSCs in the bone marrow of competitively transplanted mice at 24 weeks post-transplant. **(E)** Percentage of CD45.1 donor LT-HSCs in the bone marrow of competitively transplanted mice at 24 weeks post-transplant.

*(n=4-6 per group; All values are mean±SD; * represents P<0.05 and ns represents non-significant)*

### 5.2.5. Co-housed mice do not show any changes in spleen size or HSPC composition during acute infections

Among different triggers, infections have been shown to induce hematopoiesis in sites other than bone marrow, such as the spleen, in order to meet the increased demand for mature blood cell production (209–212). This phenomenon is called extramedullary hematopoiesis. Since there was no changes observed in the size of the HSPC compartment in the bone marrow, I assessed the spleen of the co-housed mice for signs of extramedullary hematopoiesis. However, there was no extramedullary hematopoiesis observed in the co-housed mice during acute infections, as indicated by the lack of change in the spleen weight or the number of white blood cells in the spleen **(Figure 5.11A-B)**. There was also no co-housing-induced differences in the number of LK cells (oligopotent progenitors) or LSK cells (HSPCs) in the spleen compared to the control mice **(Figure 5.11C-D)**. Furthermore, I assessed the proportion of the different oligopotent progenitors within the LK cellular compartment to understand if there were any specific differentiation trajectories. However, the proportions of CMPs, GMPs and MEPs in the spleen of co-housed mice remained the same as that of the clean control mice. This suggests that at the acute 2 month's time-point, there is no involvement of the spleen in extramedullary hematopoiesis to support the increased peripheral blood mature cell counts to fight the acute infections transferred during co-housing.

## 5.3. Assessing the chronic effects of horizontal transmission and colonization of wild microbiota in clean laboratory mice

Chronic infections of mice with highly purified pathogens have been previously shown to have a pronounced detrimental effect on hematopoiesis, extramedullary hematopoiesis and HSC fitness (30,76,77). In the previous section, the acute effects of co-housing mediated transmission of infections was studied. As the next step, I assessed the effects of long-term infection and colonization of clean laboratory mice with wild microbiota over a period of 6 months to compare and contrast the effects relative to the acute 2-months timepoint. Similar to the previous section, the co-housed mice were assessed for changes in the peripheral blood mature cell counts, bone

marrow HSPC and oligopotent progenitor compartment, as well as assessment of HSC fitness by transplantation, and determination of whether extramedullary hematopoiesis was elevated in the spleen.

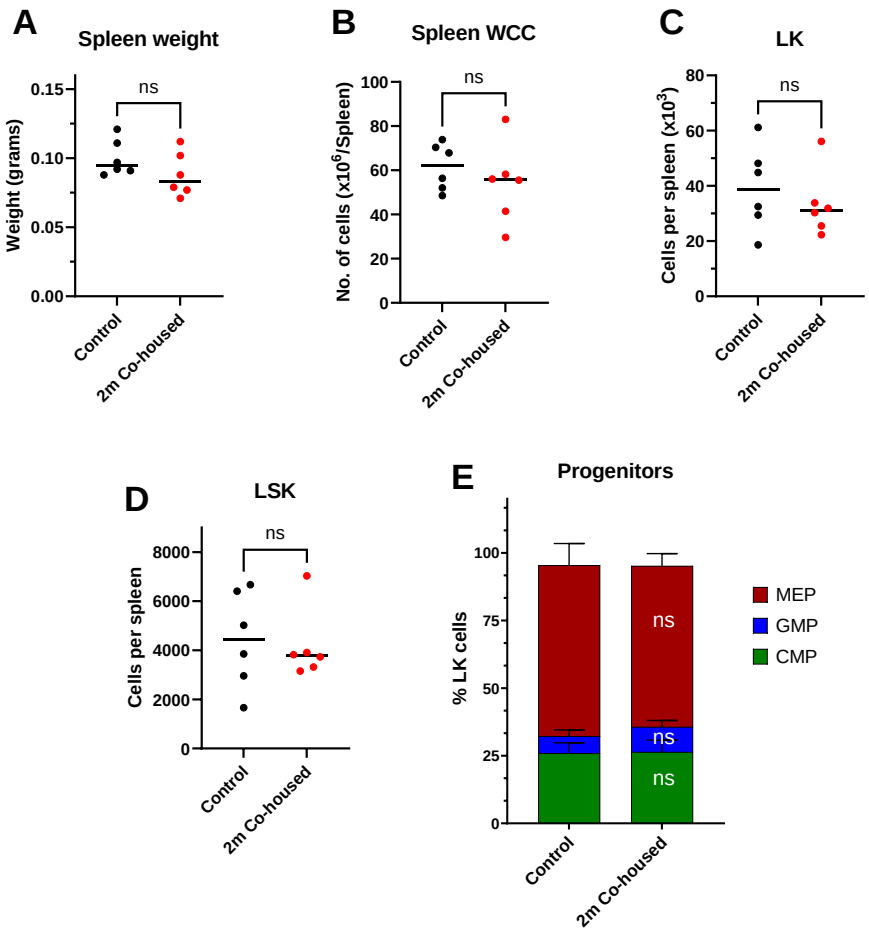


**Figure 5.11: Acute transfer of wild microbiota does not show any changes in spleen size or spleen HSPC composition**

**(A)** Spleen weight at 2 months after co-housing. **(B)** Number of white blood cells in the spleen at 2 months after co-housing. **(C)** Number of LK cells in the spleen at 2 months after co-housing. **(D)** Number of LSK cells in the spleen at 2 months after co-housing. **(E)** Proportion of MEPs, CMPs, and GMPs within the LK cell compartment in the spleen at 2 months after co-housing.
*(n=4-6 per group; All values are mean±SD; ns represents non-significant)*

### 5.3.1. Co-housed mice show sustained high peripheral blood cell counts during the long-term colonization with wild microbiota

Firstly, I wanted to understand whether the co-housing induced infections only elicit acute short-term immune response in mice or whether the response is chronically

sustained due to the number/persistence of infections. To this end, 6 months from the start of co-housing, blood samples were collected from the co-housed mice. The co-housed mice continued to show elevated overall peripheral blood lymphocyte, monocyte and granulocyte counts, even at 6 months after co-housing **(Figure 5.12A-D)**. This suggests that the mice are still mounting an active immune response against the different infections transmitted during the co-housing period. The model can therefore be used to study the chronic effects of colonization of a range of pathogens. There was no change in the red blood cell count in the peripheral blood **(Figure 5.12E)**. However, contrary to the 2 month's time-point, the co-housed mice had slightly elevated platelet counts at 6 months **(Figure 5.12F)**. This is surprising and contradictory to existing literature where viral infections have been shown to be associated with thrombocytopenia rather than thrombocytosis (213).

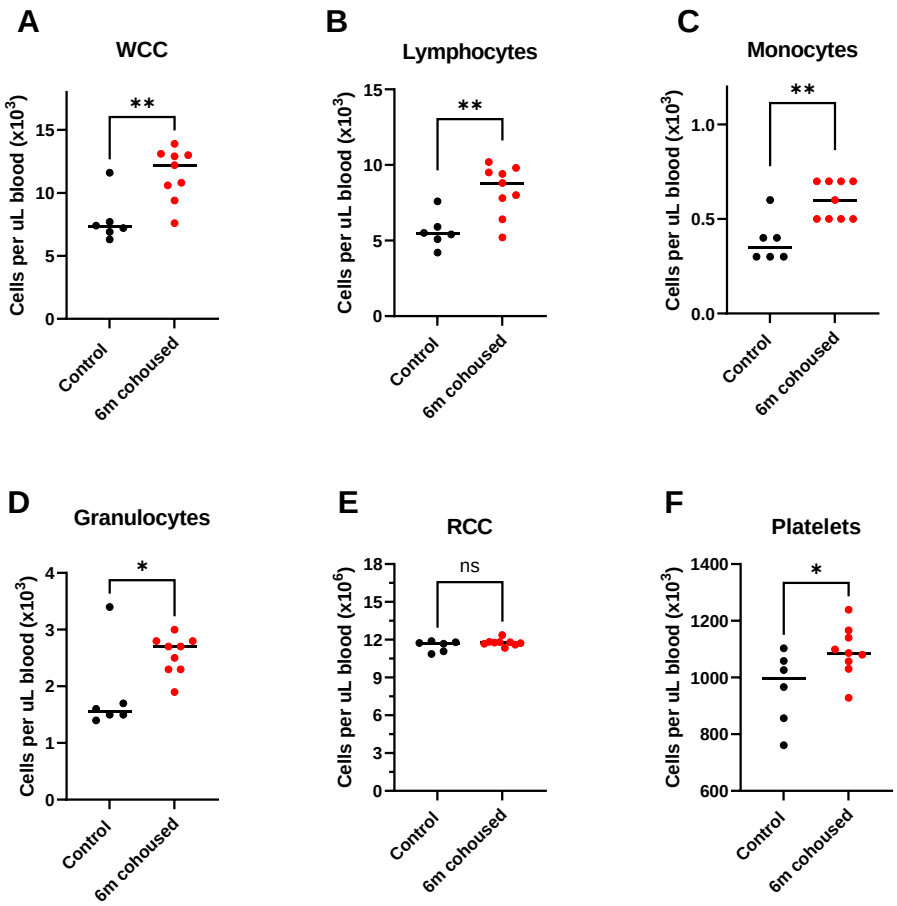


**Figure 5.12 Co-housed mice show elevated blood cell counts even after 6 months from start of co-housing**

**(A)** Peripheral blood white blood cell count at 6 months after co-housing. **(B)** Peripheral blood lymphocyte count at 6 months after co-housing. **(C)** Peripheral blood monocyte count at 6 months of co-housing. **(D)** Peripheral blood granulocyte counts at 6 months of co-housing. **(E)** Peripheral

blood red blood cell count at 6 months of co-housing. **(F)** Peripheral blood platelet count at 6 months of co-housing.
*(n=6-9 per group; All values are mean±SD; * represents P<0.05, ** represents P<0.01 and ns represents non-significant)*

Similar to the flow cytometry analysis performed on the 2 month co-housed mice, the number of mature CD11b+ myeloid cells in the peripheral blood continued to be at an elevated level at 6 months of co-housing **(Figure 5.13A)**. This was also concomitant with a significantly increased number of $Ly6C^{High}$ and $Ly6C^{Low}$ monocytes in the peripheral blood **(Figure 5.13B-C)**. This sustained increase in pro-inflammatory and patrolling monocytes, suggests that the innate immune response against the colonized infections from the co-housing is sustained. The chronically elevated number of inflammatory monocytes in the peripheral blood could have further effects on hematopoiesis higher up in the hematopoietic hierarchy. Similarly, the cells of the adaptive immune response such as B-cells, CD4+ T-cells and CD8+ T-cells also showed increased numbers in the peripheral blood of the 6 months co-housed mice **(Figure 5.13D-F)**. This again reiterates a sustained innate and adaptive immune response in the co-housed mice even at 6 months after infection transmission, validating the model to study chronic effects of infections.

### 5.3.2. Co-housed mice do not exhibit altered progenitor numbers in the bone marrow even after 6 months of sustained high peripheral blood counts

Since the peripheral blood cell count was found to be continually elevated in the co-housed mice, I next assessed if there is any expansion or reduction of the oligopotent progenitor cell compartment. Interestingly, even with the sustained high demand for production of peripheral blood mature cells, there was no significant co-housing induced changes observed in the absolute number of CMPs, CD55+ CMPs, GMPs, MEPs, or CLPs in the bone marrow **(Figure 5.14A-E)**. Such a lack of phenotype could be due to a couple of reasons. There could be more committed progenitors or precursor cells downstream of the oligopotent progenitors which are driving the mature cell production. However, this would eventually lead to an exhaustion of these precursor cells as they are not capable of self-renewal. Hence at the chronic but lower level of immune response here, it might take longer for the oligopotent progenitors to be required to start differentiating. Another reason might

be that any drop in progenitor numbers leads to the differentiated oligopotent progenitor being replaced by a new progenitor cell, thereby maintaining a homeostatic number of progenitor cells in the bone marrow. There was also no changes observed in the proportion of CMPs, GMPs or MEPs within the LK cellular compartment **(Figure 5.14F)**, meaning a lack of a specific differentiation trajectory of the oligopotent progenitors.

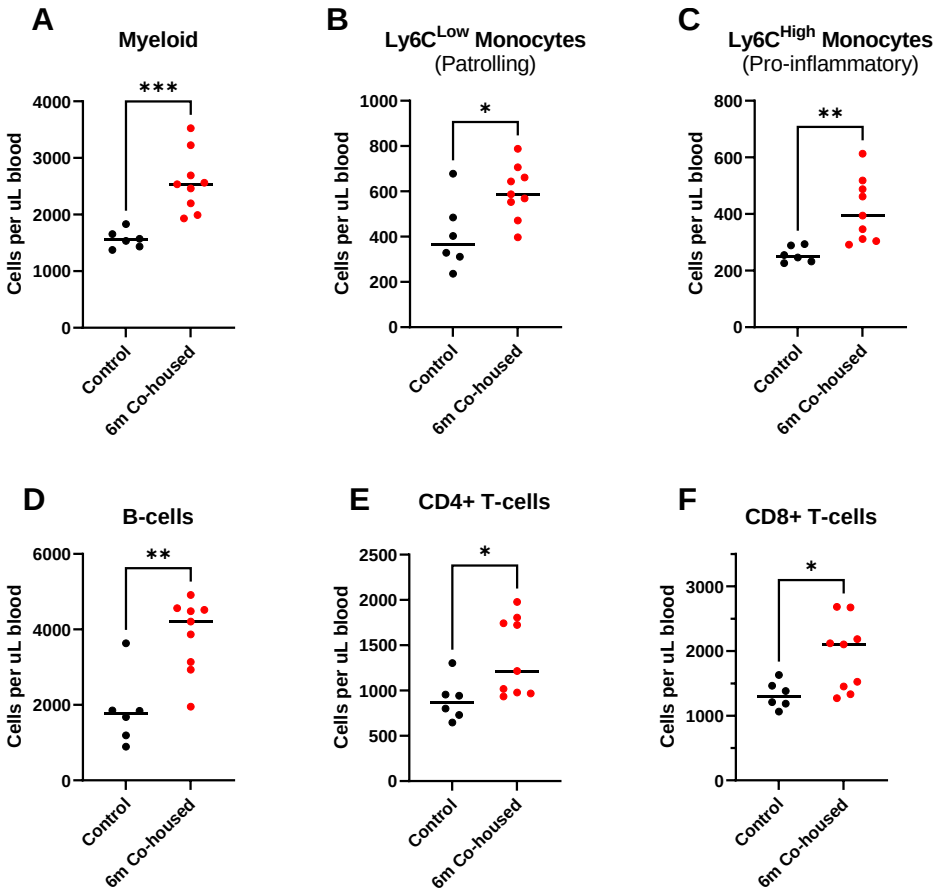


**Figure 5.13: Co-housed mice show elevated peripheral blood mature cells even after 6 months from the start of co-housing**

**(A)** Peripheral blood myeloid cell count at 6 months after co-housing. **(B)** Peripheral blood patrolling monocyte count at 6 months after co-housing. **(C)** Peripheral blood pro-inflammatory monocyte count at 6 months of co-housing. **(D)** Peripheral blood B-cell counts at 6 months of co-housing. **(E)** Peripheral blood CD4+ T-cell count at 6 months of co-housing. **(F)** Peripheral blood CD8+ T-cell count at 6 months of co-housing.

*(n=6-9 per group; All values are mean±SD; * represents P<0.05, ** represents P<0.01, *** represents P<0.001 and ns represents non-significant)*

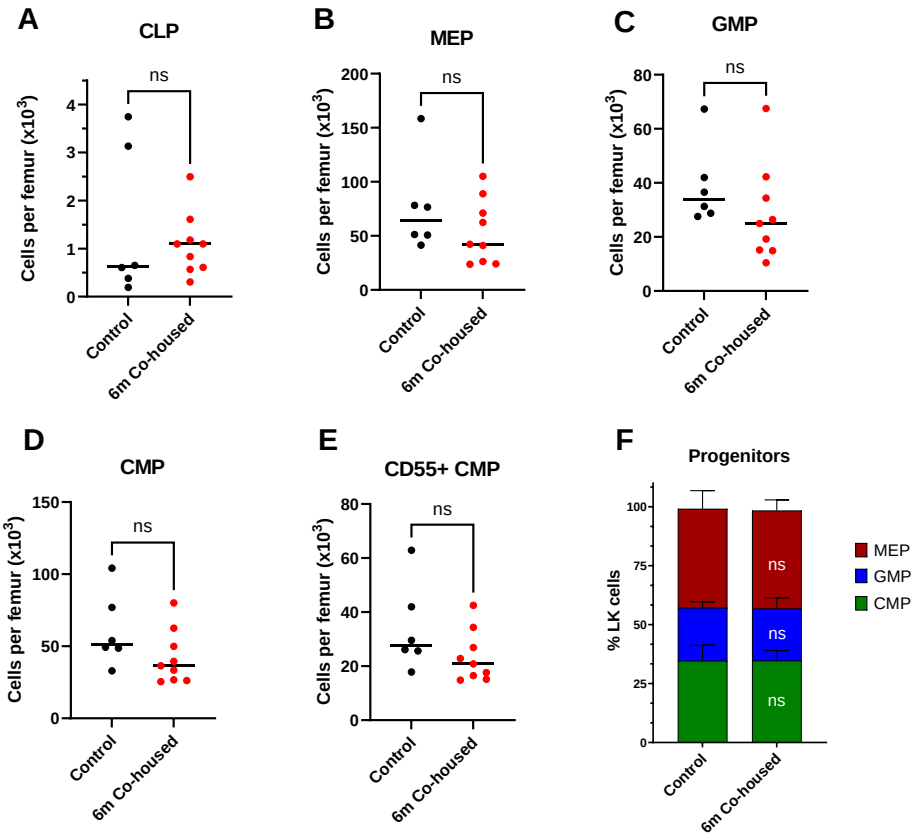


**Figure 5.14: Co-housed mice do not exhibit any changes in the numbers of different types of committed progenitor cells in the bone marrow even after sustained high peripheral blood mature cell counts**

**(A)** Number of common lymphoid progenitor (CLP) cells in the bone marrow at 6 months after co-housing. **(B)** Number of megakaryocyte-erythrocyte progenitor (MEP) cells in the bone marrow at 6 months after co-housing. **(C)** Number of granulocyte-monocyte progenitor (GMP) cells in the bone marrow at 6 months after co-housing. **(D)** Number of common myeloid progenitor (CMP) cells in the bone marrow of 6 months co-housed mice. **(E)** Number of CD55+ CMPs in the bone marrow at 6 months after co-housing. **(F)** Proportion of MEPs, CMPs, and GMPs within the LK cell compartment in the bone marrow at 6 months after co-housing.

*(n=6-9 per group; All values are mean±SD; ns represents non-significant)*

### 5.3.3. Co-housed mice show small reduction in the number of HSPCs in the bone marrow after 6 months of sustained high peripheral blood counts

Similar to the analysis performed for the 2 months co-housed mice, I also assessed the number of different HSPCs in the bone marrow of the 6 months co-housed mice. Looking at the multipotent progenitor cells (MPPs); not all the MPP subtypes showed differences in terms of absolute numbers in the bone marrow of the co-housed

mice **(Figure 5.15A-D)**. However, there was a significant reduction in the number of MPP2 and MPP4 cells in the bone marrow of the 6 months co-housed mice **(Figure 5.15A&C)**. MPP2 cells are more multipotent and have been shown to differentiate into both myeloid and lymphoid cells, while MPP4 cells have been shown to be more lymphoid biased in differentiation (208). The reduction in their numbers would indicate a differentiation of these MPPs to meet the demands of maintaining the peripheral blood mature cells, while not being able to self-renew like HSCs. The MPP2 cells might be involved in maintaining the peripheral blood myeloid cell demand and the MPP4 cells in maintaining lymphocyte numbers. However, it is interesting that the number of myeloid biased MPP3 cells are unchanged considering the increased number of myeloid cells in the peripheral blood of co-housed mice. Furthermore, there were no changes in the number of LT- or ST-HSCs in the bone marrow of the 6 months co-housed mice, despite the chronic presence of the infections **(Figure 5.15E-F)**.

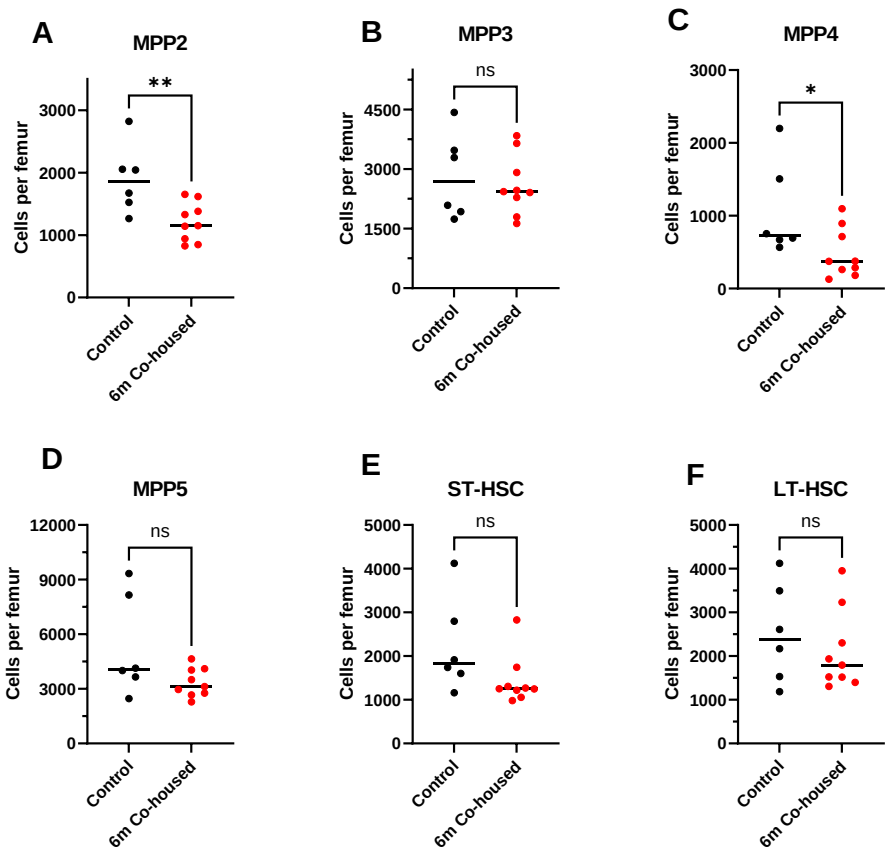


**Figure 5.15: Co-housed mice exhibit a small reduction in the number of some HSPCs in the bone marrow after 6 months from the start of co-housing**

**(A)** Number of MPP2 cells in the bone marrow at 6 months after co-housing. **(B)** Number of MPP3 cells in the bone marrow at 6 months after co-housing. **(C)** Number of MPP4 cells in the bone marrow at 6 months after co-housing. **(D)** Number of MPP5 cells in the bone marrow of 6

months co-housed mice. **(E)** Number of ST-HSCs in the bone marrow at 6 months after co-housing. **(F)** Number of LT-HSCs in the bone marrow at 6 months after co-housing.
*(n=6 per group; All values are mean±SD; ns represents non-significant)*

This is in striking contrast to other more severe models of infections, where the number of LT-HSCs in the bone marrow has been shown to drastically reduce during chronic infections (30). This shows that under both acute and chronic colonization of wild microbiota into clean laboratory mice, there is no expansion or depletion of the absolute number of immunophenotypically-defined HSCs.

### 5.3.4. Competitive transplantation assay shows that chronic infections from wild microbiota reduces long-term repopulation potential of HSCs

Even though there were no differences in the absolute number of HSCs in the bone marrow of co-housed mice, I wanted to assess whether there are any functional defects in the HSCs as previously shown in other models of chronic infections. To this end, I performed a competitive transplantation assay with bone marrow from 6 months co-housed mice, as previously performed for the 2 months co-housed mice **(Figure 5.10A)**. It was observed that there was a significantly reduced percentage of CD45.1 donor cells (6 months co-housed) in the peripheral blood of recipient mice starting from 12 weeks post-transplant **(Figure 5.16A)**. This means that the sustained chronic infections from the wild microbiota led to a reduction in the functional potency of the HSCs. To further understand if the reduced engraftment is the same across all the mature blood lineages, I also assessed the proportion of CD45.1 cells within the different mature cell populations. The peripheral blood had significantly reduced contribution from the co-housed mouse donor HSCs across both the myeloid and the lymphoid lineages (CD4+ T-cells, CD8+ T-cells and B-cells) at 12 weeks post-transplant **(Figure 5.16B-E)**. This reduced engraftment continued to be low within the lymphoid lineage up to 24 weeks post-transplant. Interestingly, the contribution of co-housed mouse HSCs towards the myeloid lineage was not found to be significantly different to that from the control bone marrow HSCs at the 24 weeks endpoint.

After looking at the peripheral blood contribution over time, I also analyzed the bone marrow HSPCs of the recipient mice at 24 weeks post-transplant. I observed that there was no significant difference in the proportion of CD45.1 LSK cells (HSPCs) in the bone marrow of mice transplanted with co-housed mouse bone marrow compared

to bone marrow from control mice **(Figure 5.16F)**. Furthermore, there was also no reduction in the percentage of CD45.1 ST- or LT-HSCs in the bone marrow of mice transplanted with co-housed mouse bone marrow **(Figure 5.16G-H)**. Hence the HSCs from co-housed mice do not have any engraftment defects. This indicates that the HSCs from long-term co-housed mice have functional defects in their ability to differentiate and repopulate the different hematopoietic lineages even though the number of immunophenotypically defined-HSCs in the bone marrow is unaltered.

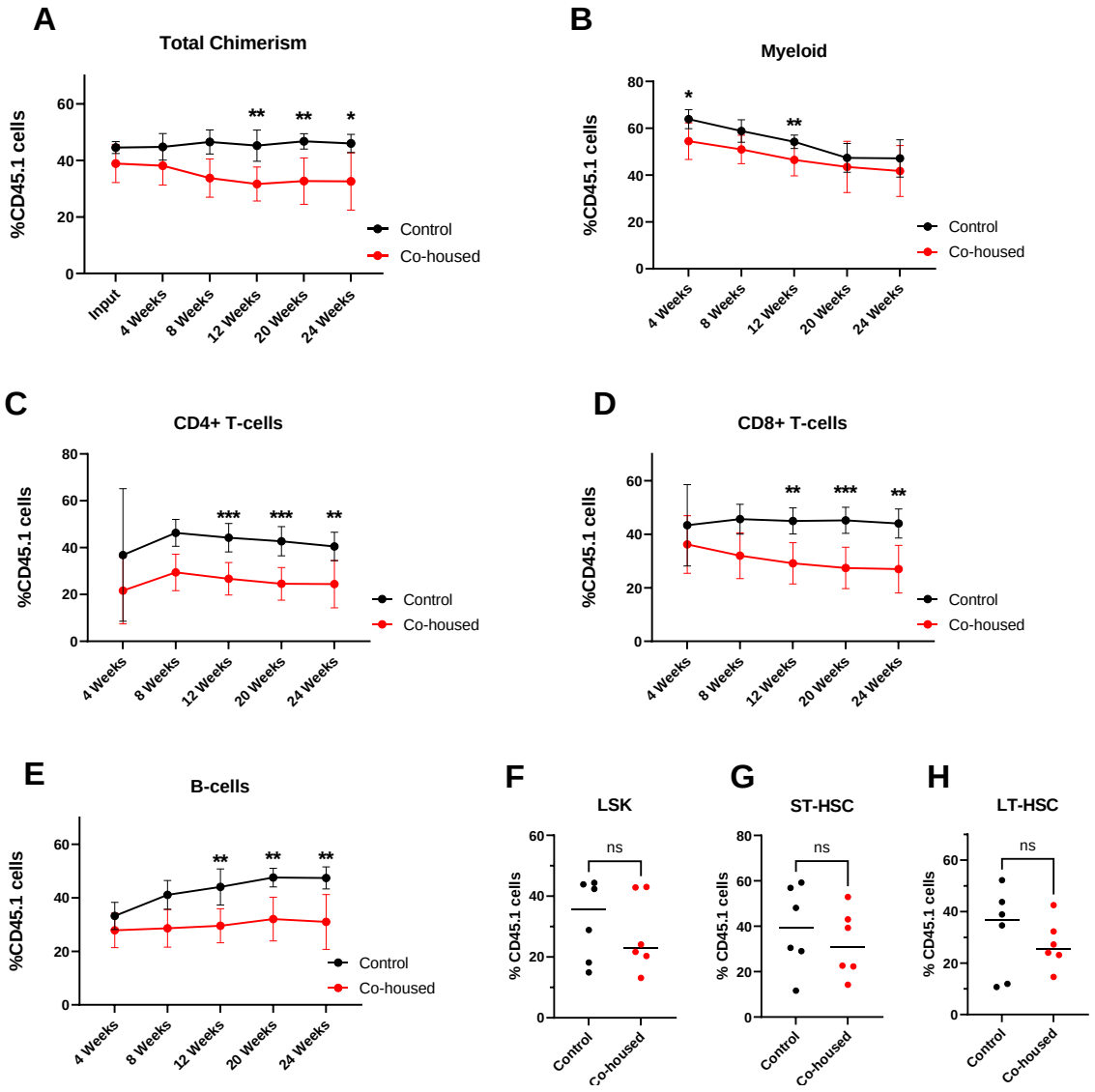


**Figure 5.16: Bone marrow from mice co-housed for 6 months show defects in long-term multi-lineage engraftment**

**(A)** Change in total peripheral blood donor (CD45.1 6 months co-housed or control mice) chimerism over 24 weeks from transplantation. **(B)** Change in peripheral blood donor (CD45.1 6 months co-housed or control mice) myeloid cell chimerism over 24 weeks from transplantation. **(C)** Change in peripheral blood donor (CD45.1 6 months co-housed or control mice) CD4+ T-cell chimerism over

24 weeks from transplantation. **(D)** Change in peripheral blood donor (CD45.1 6 months co-housed or control mice) CD8+ T-cell chimerism over 24 weeks from transplantation. **(E)** Change in peripheral blood donor (CD45.1 6 months co-housed or control mice) B-cell chimerism over 24 weeks from transplantation**. (F)** Percentage of CD45.1 donor LSK cells in the bone marrow of competitively transplanted mice at 24 weeks post-transplant. **(G)** Percentage of CD45.1 donor ST-HSCs in the bone marrow of competitively transplanted mice at 24 weeks post-transplant. **(H)** Percentage of CD45.1 donor LT-HSCs in the bone marrow of competitively transplanted mice at 24 weeks post-transplant.
*(n=6 per group; All values are mean±SD; * represents P<0.05, ** represents P<0.01, *** represents P<0.001 and ns represents non-significant)*

### 5.3.5. Co-housing mediated reduction in HSC fitness is not concomitant with any changes in HSC dormancy or proliferation

Previous studies showing loss of functional HSCs in mice have been linked to the push of dormant HSCs into active proliferation (27,28). To this end, I performed a BrdU pulse-chase experiment along with co-housing **(Figure 5.17A)**. Wildtype SPF mice were administered with BrdU for a period of 14 days to label the HSCs with BrdU. Following the BrdU treatment, the mice were co-housed with a wildling mouse in the same cage for a period of 3 weeks and the wildling mouse was removed. Mice that were housed under SPF conditions in the absence of co-housing were used as controls. The bone marrow of the co-housed mice were then analyzed at 2 different timepoints (4 weeks and 12 weeks) from the start of co-housing to assess the proportion of label-retaining dormant HSCs through flow cytometry. There was no significant difference observed in the proportion of BrdU+ label-retaining dormant LT-HSCs in the bone marrow of co-housed mice compared to that in the clean control mice **(Figure 5.17B)**. This would indicate that co-housing mediated infections or wild microbiota colonization does not drive dormant HSCs more into proliferation. Thus any functional defect observed in the HSCs from long-term co-housed mice might not be due to increased proliferation as has been the case in previous studies on infection-mediated loss of HSC fitness. However, it has to be noted here that the initial BrdU labeling is dependent on cell proliferation and any deeply dormant HSCs are not labelled by BrdU to begin with. The proliferation of these deeply dormant fraction of LT-HSCs could be contributing to the functional defects observed, and hence cannot be fully assessed with this BrdU-labelling model system used here. Hence, a previously published genetic mouse model to assess HSC dormancy and proliferative index (25), was tested. These transgenic mice have H2B-GFP expression in all HSCs and the expression of

H2B-GFP can be switched off by treating the mice continuously with doxycycline, to enable a label-chase similar to BrdU. This would ideally eliminate the major problem associated to inefficient pulse-labelling with the BrdU system. However, it was observed in initial pilot experiments that the doxycycline treatment completely eliminated the bacterial arm of the wild microbiota in the co-housed and the wildling mice. This removes a significant portion of the microbial transfer which was aimed to be achieved with co-housing. Therefore, this model was not further used to assess HSC dormancy with the co-housing system.

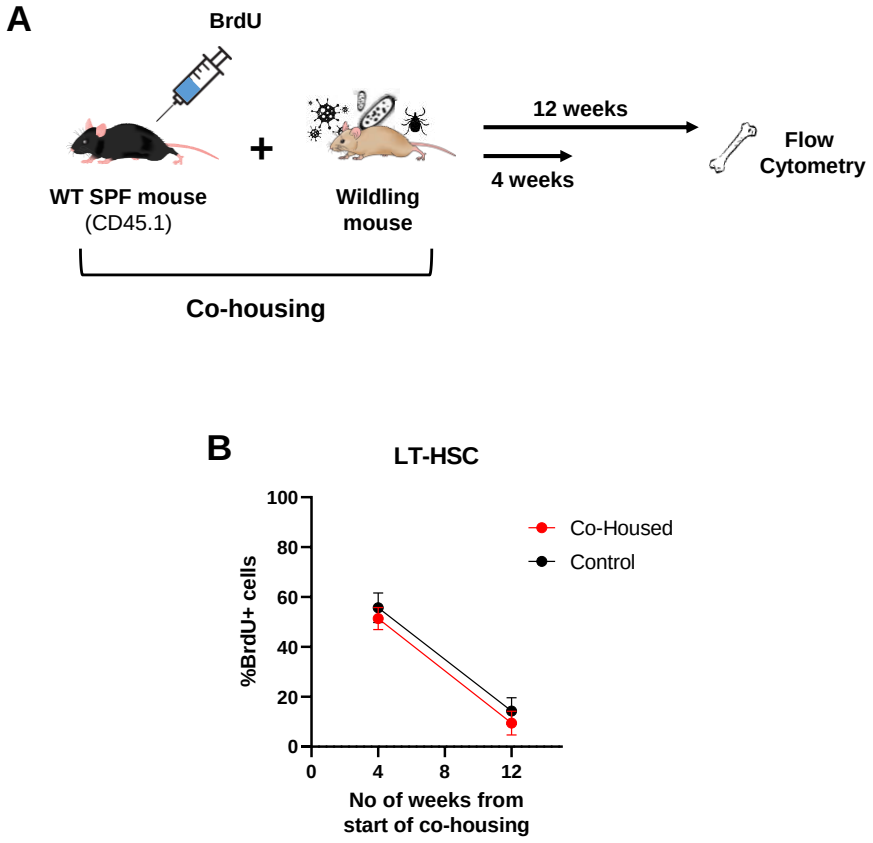


**Figure 5.17: Co-housed mice do not have any differences in the proportion of dormant LT-HSCs upto 12 weeks from the start of co-housing**

**(A)** Experimental scheme for BrdU pulse-chase experiment to assess HSC dormancy in co-housed mice. **(B)** Change in percentage of BrdU+ dormant LT-HSCs in the bone marrow of co-housed mice.

*(n=6 per group; All values are mean±SD)*

### 5.3.6. Long-term co-housed mice have reduced spleen size and reduced number of oligopotent progenitors and HSPCs

Having established that acute effects of co-housing mediated infections does not involve any changes in the spleen, I wanted to assess and compare any effects in the spleen after chronic infections at 6 months of co-housing. Hence the spleen of the co-housed mice after 6 months was analyzed. The spleen of the co-housed mice were found to be smaller in size with reduced weight and also reduced overall number of white blood cells compared to that of clean control mice **(Figure 5.18A-B)**. To further study if this reduction in spleen size and cell count was due to impaired extramedullary hematopoiesis, I also measured the number of oligopotent progenitors and HSPCs in the spleen through flow cytometry. The reduced spleen white cell count was concomitant with a reduction in the number of LK cells (Oligopotent progenitors) and LSK cells (HSPCs) in the spleen **(Figure 5.18C-D)**. This reduction in spleen progenitors and HSPCs could be due to increased hematopoietic differentiation in the spleen, thereby leading to a HSPC exhaustion phenotype within the spleen hematopoietic niche. The increased differentiation of HSPCs in the spleen potentially contributing to the mature peripheral blood cell production, could explain the lack of any change in the number of progenitors or HSPCs in the bone marrow of these mice. However, a lack of expansion of any of these progenitor cells in the spleen would suggest that at the time-point of analysis, there was a lack of mobilization of HSPCs from the bone marrow into the spleen, or alternatively a reduced proliferation of spleen resident HSPCs. These non-mutually exclusive processes could mediate the drop in number of HSPCs in the spleen and might be more prominent at an even longer time post co-housing mediated infection response. However, there was no differences observed in the long term co-housed mice in terms of proportions of the different oligopotent progenitors (CMPs, GMPs and MEPs) **(Figure 5.18E)**, indicating no differentiation bias in the spleen HSPCs.

## 5.4. Assessing the effects of vertical neonatal transfer of wild microbiota on hematopoiesis in the wildling mice

Co-housing wildtype SPF mice with the wildling mice is a nice model system to study the effects of natural horizontal transmission of infections on the hematopoietic system. It has enabled me to assess the cells of the different layers of the hematopoietic

hierarchy responding to both the acute transfer of infections and long term colonization of the infections/microbiota. However, direct assessment of wildling mice gave me the additional opportunity to further assess how vertical transmission of infections affects steady state hematopoiesis. To this end, I performed all the analysis as performed for the co-housed mice on the peripheral blood, bone marrow and spleen. The wildling mice analyzed were the same mice used for the co-housing and were age-matched to the 6 months co-housed mice. Age-matched SPF C57BL6/NTac mice were used as controls. It is to be noted here however that the wildling mice are of the C57BL6/Ntac strain and could be slightly different to the C57BL6/J mice used for the co-housing. Hence all comparisons of wildling mice were made relative to SPF C57BL6/NTac control mice.

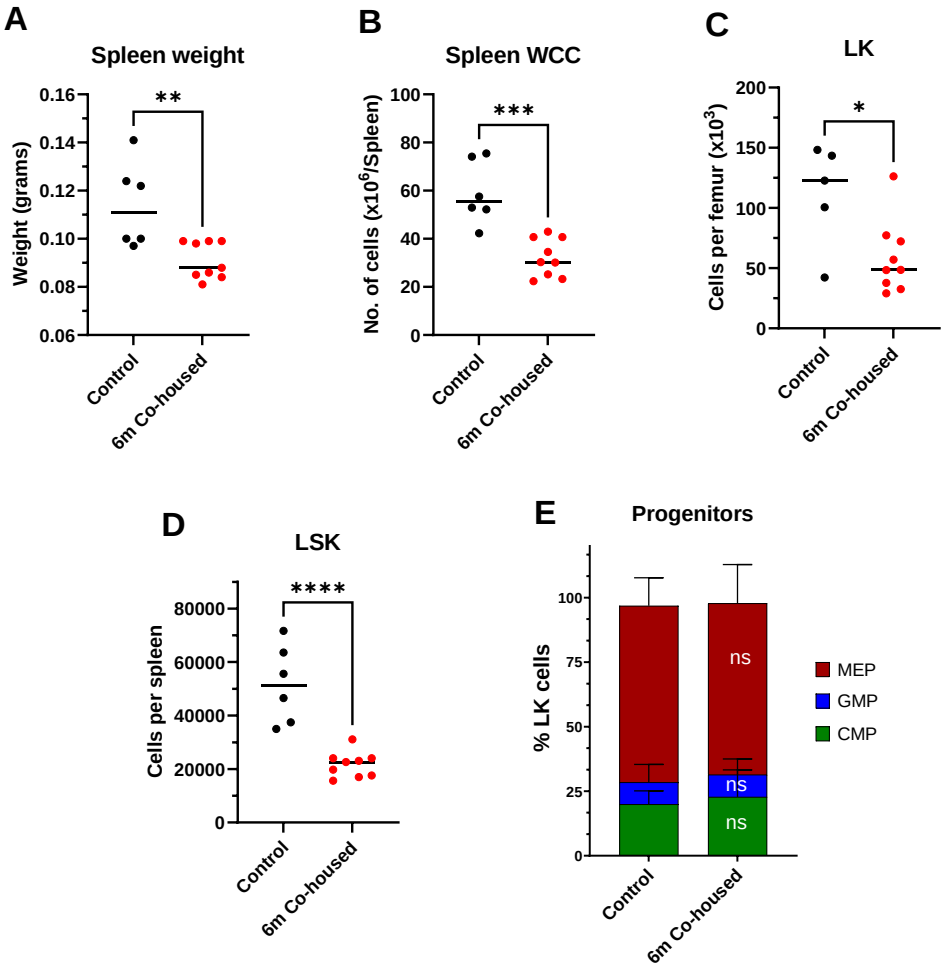


**Figure 5.18: Co-housed mice show reduced spleen size and reduced number of commited progenitors and HSPCs after 6 months of co-housing**

**(A)** Spleen weight at 6 months after co-housing. **(B)** Number of white blood cells in the spleen at 6 months after co-housing. **(C)** Number of LK cells in the spleen at 6 months after co-housing. **(D)**

Number of LSK cells in the spleen at 6 months after co-housing. **(E)** Proportion of MEPs, CMPs, and GMPs within the LK cell compartment in the spleen at 6 months after co-housing.
*(n=6-9 per group; All values are mean±SD; * represents P<0.05, ** represents P<0.01, *** represents P<0.001, **** represents P<0.0001 and ns represents non-significant)*

### 5.4.1. Wildling mice do not show major differences in the number of mature cells in the peripheral blood

Firstly, I wanted to assess the peripheral blood cell counts of the wildling mice to understand if the transfer of wild microbiota during birth/weaning has the same effects on the peripheral blood as co-housing of adult mice. Hence, blood samples were collected from the wildling mice at the same age (8 months), as the sampling was performed from the mice that were co-housed for 6 months. Surprisingly, the overall peripheral blood white cell count was not different from the clean SPF control mice **(Figure 5.19A)**. There was also no elevated number of lymphocytes observed in the peripheral blood as was seen in the co-housed mice **(Figure 5.19B)**. The number of monocytes and granulocytes was slightly elevated in the wildling mice compared to the clean control mice **(Figure 5.19C-D)**. However, the increase was subtle and much less than that observed in the co-housed mice. The red cell counts was noted to be significantly increased in the wildling mice **(Figure 5.19E)**, which was not the case in the co-housed mice. However, the biological significance of increased erythrocytes in the peripheral blood is not clear. There was no differences observed in the peripheral blood platelet count **(Figure 5.19F)**.

Since there were no changes in the overall peripheral blood cell counts of the wildling mice, I further assessed the number of different subsets of mature cells in the periphery using flow cytometry, analogous to the characterization of blood in the co-housed mice. Again, I observed no differences in the number of overall blood myeloid cells or the $Ly6C^{Low}$ or $Ly6C^{High}$ monocytes in the wildling mice compared to control mice **(Figure 5.20A-C)**. Similarly, there was also no difference observed in the number of different lymphoid cells in the peripheral blood **(Figure 5.20D-F)**. Overall, these data suggest that the peripheral blood cell counts of the wildling mice are similar to their clean counterparts, unlike what was observed during co-housing. The lack of elevated mature blood cell counts is especially surprising since the wildling mice at this age were found to have active infections, as evidenced by the microbiological analysis of these mice **(Figure 5.1)**. Hence, this would indicate colonization by wild

microbiota and infections during or shortly after birth somehow prevents the peripheral blood phenotypes that are generated as a result of horizontal transfer of the same microorganisms/pathogens during co-housing of adult mice.

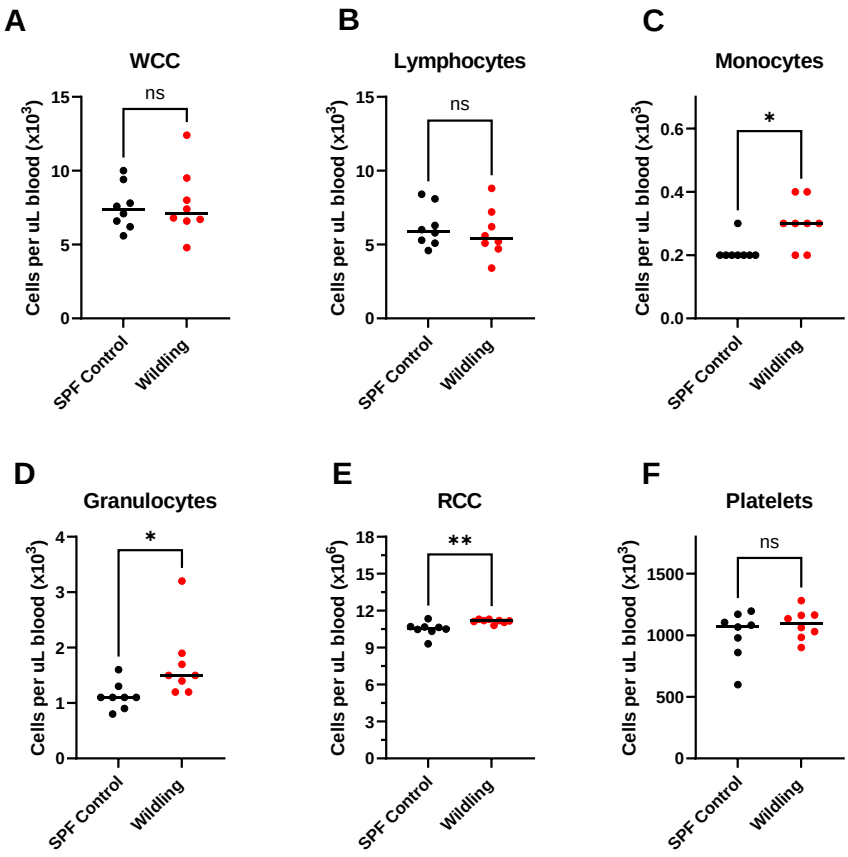


**Figure 5.19: Wilding mice show mostly normal peripheral blood cell counts**
**(A)** Peripheral blood white blood cell count in wildling and control mice. **(B)** Peripheral blood lymphocyte count in wildling and control mice. **(C)** Peripheral blood monocyte count in wildling and control mice. **(D)** Peripheral blood granulocyte counts in wildling and control mice. **(E)** Peripheral blood red blood cell count in wildling and control mice. **(F)** Peripheral blood platelet count in wildling and control mice.
*(n=8 per group; All values are mean±SD; * represents P<0.05, ** represents P<0.01 and ns represents non-significant)*

Since the wildling mice do not show any differences in the peripheral blood cell counts at the same age as the 6 months co-housed mice, I wanted to understand if there was any immune response to the infections present in the wildling mice. For this, I analyzed the proportions of the different CD4+ and CD8+ T-cell subsets using flow cytometry. It was observed that the wildling mice had increased proportion of active

CD4+ T-cells **(Figure 5.21A-B)**. They also exhibited an elevated proportion of CD8+ memory T-cells **(Figure 5.21C-D)**. Both these observations are in line with that observed in the co-housed mice after the co-housing phase, indicating a current and a past adaptive immune response against the infections.

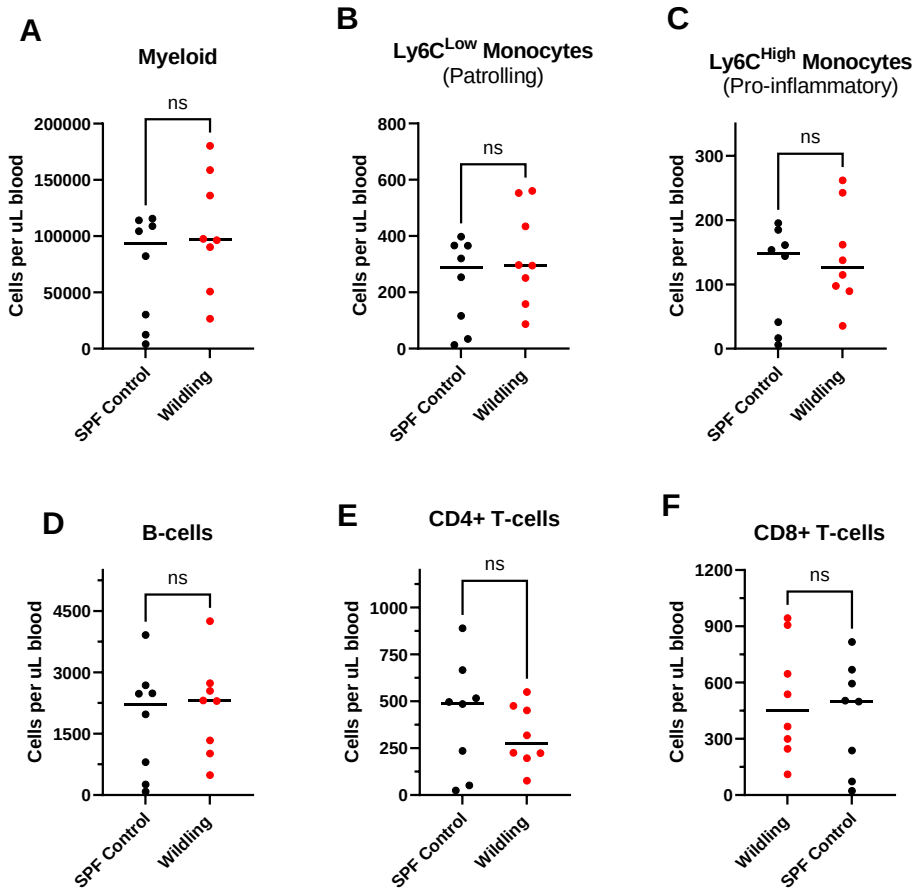


**Figure 5.20: Wilding mice do not show changes in the number of mature cells in the peripheral blood at 8 months of age**

**(A)** Peripheral blood myeloid cell count in wildling and control mice. **(B)** Peripheral blood patrolling monocyte count in wildling and control mice. **(C)** Peripheral blood pro-inflammatory monocyte count in wildling and control mice. **(D)** Peripheral blood B-cell counts in wildling and control mice. **(E)** Peripheral blood CD4+ T-cell count in wildling and control mice. **(F)** Peripheral blood CD8+ T-cell count in wildling and control mice.

*(n=8 per group; All values are mean±SD; ns represents non-significant)*

In conclusion, the wildling mice have normal peripheral blood counts in spite of an immune response against the colonized infections. The homeostatic peripheral blood count values are therefore not different to that of SPF control mice, when the

wild microbiota colonization occurs earlier in the lifetime (neonatal) of the mice compared to acquiring them during adulthood in the co-housed mice.

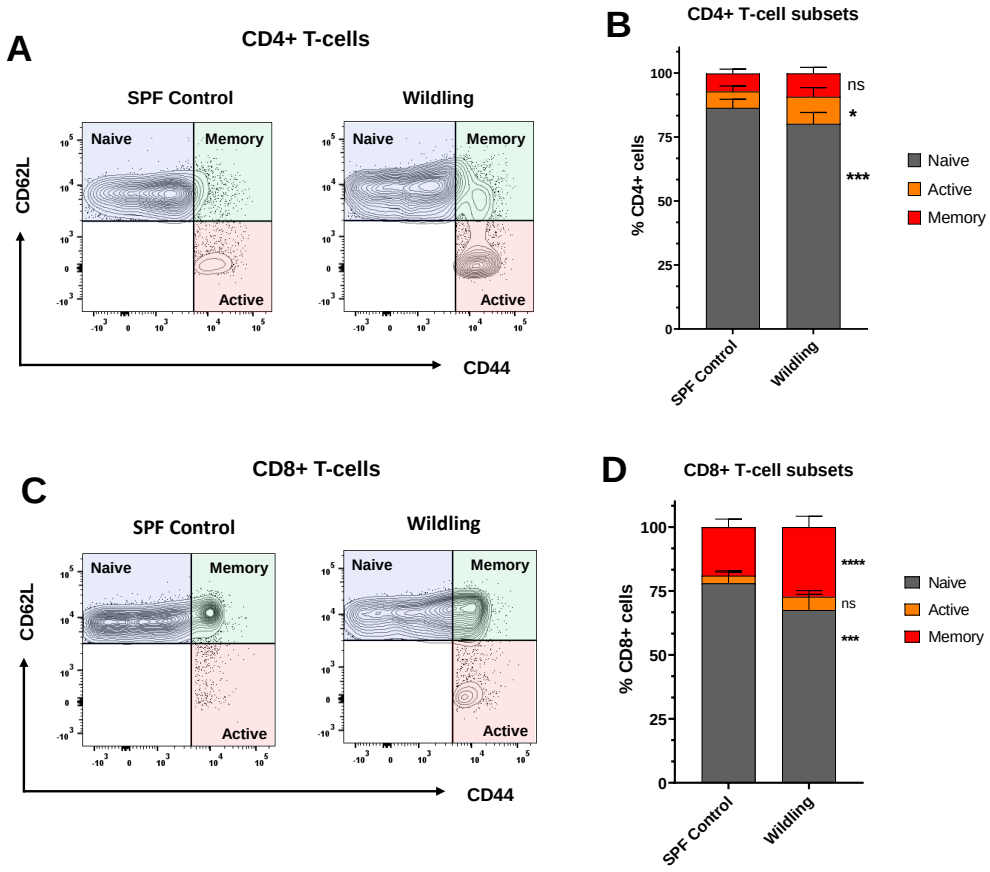


**Figure 5.21: Wilding mice have altered proportions of active and memory T-cells in the peripheral blood**

**(A)** Representative flow cytometry plot representing the different CD4+ T-cell subsets. **(B)** Proportions of the different CD4+ T-cell subsets in the peripheral blood of wildling and control mice. **(C)** Representative flow cytometry plot representing the different CD8+ T-cell subsets. **(D)** Proportions of the different CD4+ T-cell subsets in the peripheral blood of wildling and control mice.

*(n=8 per group; All values are mean±SD; * represents P<0.05, *** represents P<0.001, **** represents P<0.0001 and ns represents non-significant)*

### 5.4.2. Wildling mice have normal numbers of oligopotent progenitors in the bone marrow

I next analyzed the oligopotent progenitor cell compartment in the bone marrow of the wildling mice, to understand if having the infection process during the neonatal period alters the basal number of these progenitors under homeostasis. There

was no differences observed in the absolute number of CLPs, CMPs, CD55+ CMPs, MEPs or GMPs in the bone marrow of the wildling mice **(Figure 5.22A-E)**. However there was trend towards increased MEPs in the wildling mice compared to the control mice **(Figure 5.22B)**, which is in line with the slightly increased erythrocyte count observed in the peripheral blood. There was no significant difference observed in the distribution of the different progenitors within the LK cellular compartment **(Figure 5.22F)**, meaning no differentiation trajectory bias in the wildling mice. Here again, there was a trend towards a subtle increase in the proportion of MEPs, the biological significance of which is unclear. However, there is some evidence in the literature relating to the role of erythrocytes in innate immunity and inflammatory responses (214). The lack of significant differences in the overall number of oligopotent progenitors in the bone marrow is similar to that observed in the 6 months co-housed mice. However, it is less surprising in this setting, since the changes in the peripheral blood cell counts are extremely modest compared to that observed in the co-housed mice.

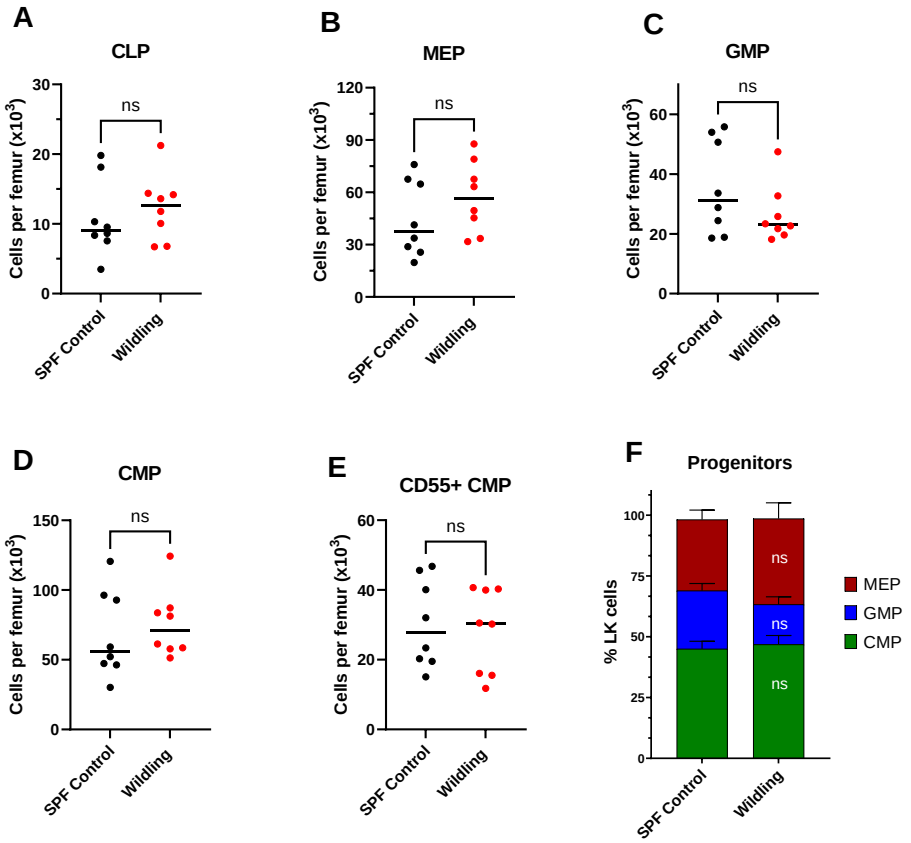


Figure 5.22: Wilding mice have normal committed progenitor numbers in the bone marrow

(A) Number of common lymphoid progenitor (CLP) cells in the bone marrow of wildling and control mice. (B) Number of megakaryocyte-erythrocyte progenitor (MEP) cells in the bone marrow of wildling and control mice. (C) Number of granulocyte-monocyte progenitor (GMP) cells in the bone marrow of wildling and control mice. (D) Number of common myeloid progenitor (CMP) cells in the bone marrow of wildling and control mice. (E) Number of CD55+ CMPs in the bone marrow of wildling and control mice. (F) Proportion of MEPs, CMPs, and GMPs within the LK cell compartment in the bone marrow of wildling and control mice.
*(n=8 per group; All values are mean±SD; ns represents non-significant)*

### 5.4.3. Wildling mice have normal numbers of HSPCs in the bone marrow

In line with the analysis performed for the co-housed mice, I also analyzed the number of HSPCs in the bone marrow of the wildling mice. There was no differences observed in the number of different MPP cell sub-types (MPP2, MPP3, MPP4 or MPP5) in the wildling mice compared to the clean control mice **(Figure 5.23A-D)**. Similarly, there was also no significant differences observed in the absolute number of ST- or LT-HSCs in the bone marrow of wildling mice **(Figure 5.23E-F)**. Both these observations are again in line with the observations made in the bone marrow HSPC numbers in the co-housed mice at the 6 month's time-point. Together, these observations make it clear that transmission of wild microbiota infections (horizontally or vertically) does not elicit any long-term changes in the absolute number of HSPCs in the bone marrow even with active infections present in the mice.

### 5.4.4. Wildling mice do not show any functional defects of HSCs in repopulating the hematopoietic system

Since the 6 months co-housed mice also showed no changes in the number of HSCs in the bone marrow but still exhibited a functional defect, I also assessed the bone marrow from wildling mice using competitive transplantation assay. To this end, I transplanted bone marrow from the CD45.2 wildling mice along with CD45.1/2 SPF competitor mouse bone marrow in a 1:1 ratio into lethally irradiated CD45.1 recipient mice **(Figure 5.24A)**. The recipient mice were bled once every 4 weeks, up to 24 weeks post-transplantation, to assess the percentage of CD45.2 donor cells in the peripheral blood. Mice transplanted with WT SPF control mouse bone marrow along with competitor bone marrow were used as controls. In contrast to the 6 months co-housed mice, the wildling bone marrow transplanted mice did not show any differences in the

proportion of CD45.2 donor cells in the peripheral blood at 24 weeks post-transplant **(Figure 5.24B)**. There was a reduced percentage of CD45.2 donor cells observed in the peripheral blood during the initial 4 week engraftment phase, but the CD45.2 donor chimerism normalized over the subsequent timepoints, similar to that of the control mice. Furthermore, I also analyzed the proportion of CD45.2 donor chimerism among the HSPCs in the bone marrow. Here again, there was no difference in terms of percentage of CD45.2 cells within the LSK (HSPCs) compartment or specifically the ST- or LT-HSCs **(Figure 5.24C-E)**.

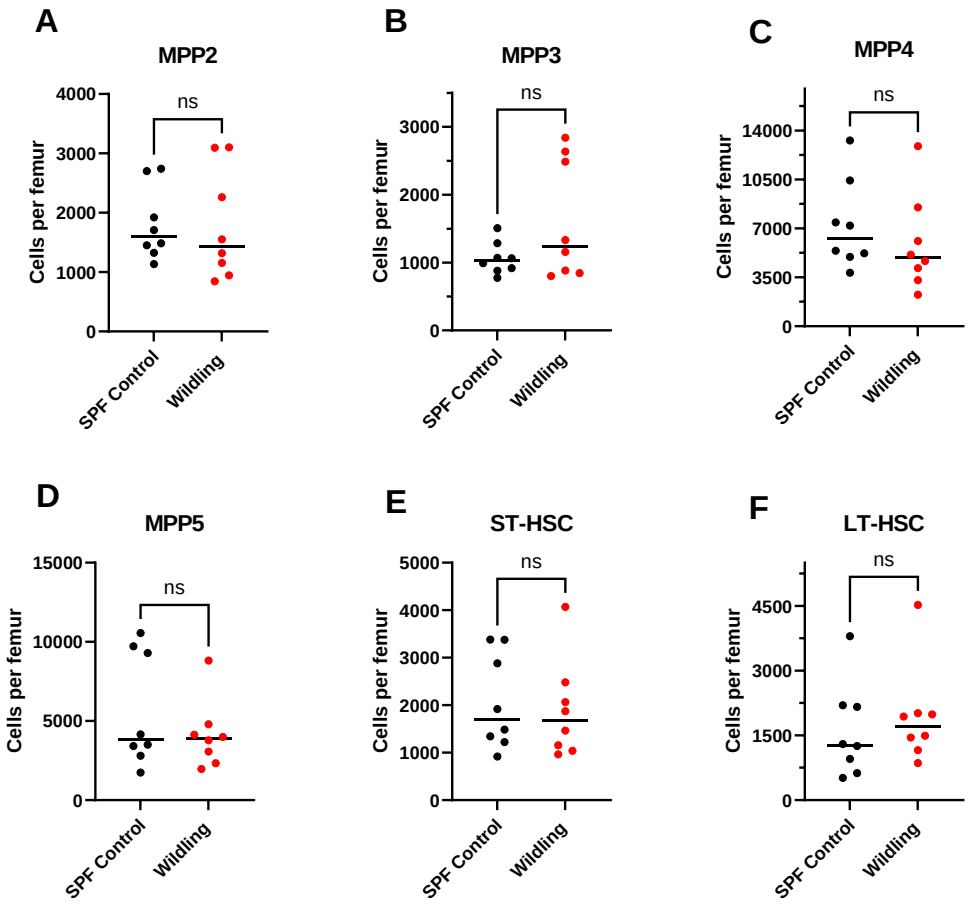


**Figure 5.23: Wilding mice have normal HSPC numbers in the bone marrow**
**(A)** Number of MPP2 cells in the bone marrow of wildling and control mice. **(B)** Number of MPP3 cells in the bone marrow of wildling and control mice. **(C)** Number of MPP4 cells in the bone marrow of wildling and control mice. **(D)** Number of MPP5 cells in the bone marrow of wildling and control mice. **(E)** Number of ST-HSCs in the bone marrow of wildling and control mice. **(F)** Number of LT-HSCs in the bone marrow of wildling and control mice.
*(n=8 per group; All values are mean±SD; ns represents non-significant)*

Overall, these observations show that the wildling mice do not have any defects in HSC engraftment or fitness relating to long-term reconstitution of the hematopoietic system following competetive transplantation, even though the wildling mice have been exposed to the wild microbiota for a longer period than to co-housed mice, since they were colonized directly during/shortly after birth. This indicates that having these infections from birth or neonatally preserves the HSC fitness through an unknown mechanism, as opposed to acquiring and sustaining these infections from adulthood.

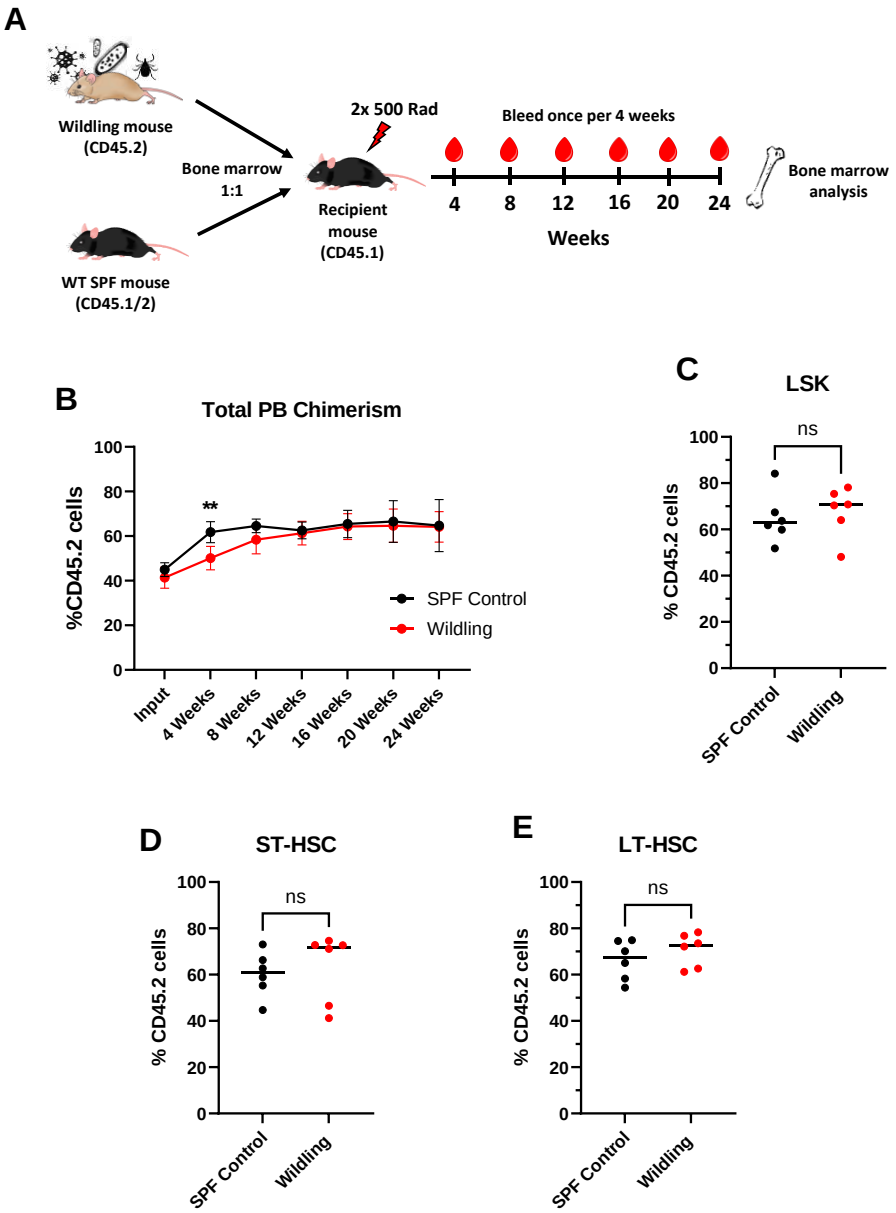


Figure 5.24: Competitive transplantation assay shows that wildling mice do not show reduced HSC fitness

**(A)** Experimental scheme for competitive transplantation assay to assess HSC functional potency. **(B)** Change in total peripheral blood donor (CD45.2 wilding or control mice) chimerism over 24 weeks from transplantation **(C)** Percentage of CD45.2 donor LSK cells in the bone marrow of competitively transplanted mice at 24 weeks post-transplant. **(D)** Percentage of CD45.2 donor ST-HSCs in the bone marrow of competitively transplanted mice at 24 weeks post-transplant. **(E)** Percentage of CD45.2 donor LT-HSCs in the bone marrow of competitively transplanted mice at 24 weeks post-transplant**.**
*(n=6 per group; All values are mean±SD; ** represents P<0.01 and ns represents non-significant)*

### 5.4.5. Wildling mice show increased proliferation of MPP3/4 cells in the bone marrow

Even though the wildling mice did not show any differences in the absolute number of oligopotent progenitors or HSPCs, there could still be increased proliferation of these cells in conjunction with increased differentiation. To assess this, I performed a BrdU incorporation assay on the wildling mice **(Figure 5.25A)**. Wilding mice were injected with BrdU in PBS (100mg/kg BW) and the mice were euthanized 16 hours after the injection. The bone marrow was then isolated and analyzed for the proportions of different oligopotent progenitors and HSPCs that are BrdU positive. In this setting, BrdU positivity indicates proliferating cells that incorporate this nucleotide analogue into their DNA following this short labelling period. Wildtype SPF C57BL6/NTac mice injected with BrdU were used a controls.

Looking at the oligopotent progenitors first, there was no differences in the proportion of BrdU positive proliferating MEPs, GMPs, or CMPs in the bone marrow of wildling mice compared to control mice **(Figure 5.25B-D)**. Thus, the oligopotent progenitors here are not driven to proliferate in response to infection/colonization. Next I analyzed the HSPC compartment and found that the MPP3/4 cells had significantly higher proportions of BrdU positive cells in the wildling mice, meaning they are actively proliferating more compared to those in the clean control mice **(Figure 5.25E)**. MPP3/4 cell population is a mixture of lymphoid and myeloid primed MPPs (208). Hence this suggests that there is a slightly increased demand on bone marrow hematopoiesis in response to the infections in the wildling mice, even if not clearly noticeable in the peripheral blood cell counts. However, the proportions of BrdU positive ST- and LT-HSCs was found to be unaltered in the wildling mice **(Figure 5.25F-G)**. This is also in line with the observation that the LT-HSCs are not

differentially proliferating in the co-housed mice in response to long-term colonization of infections.

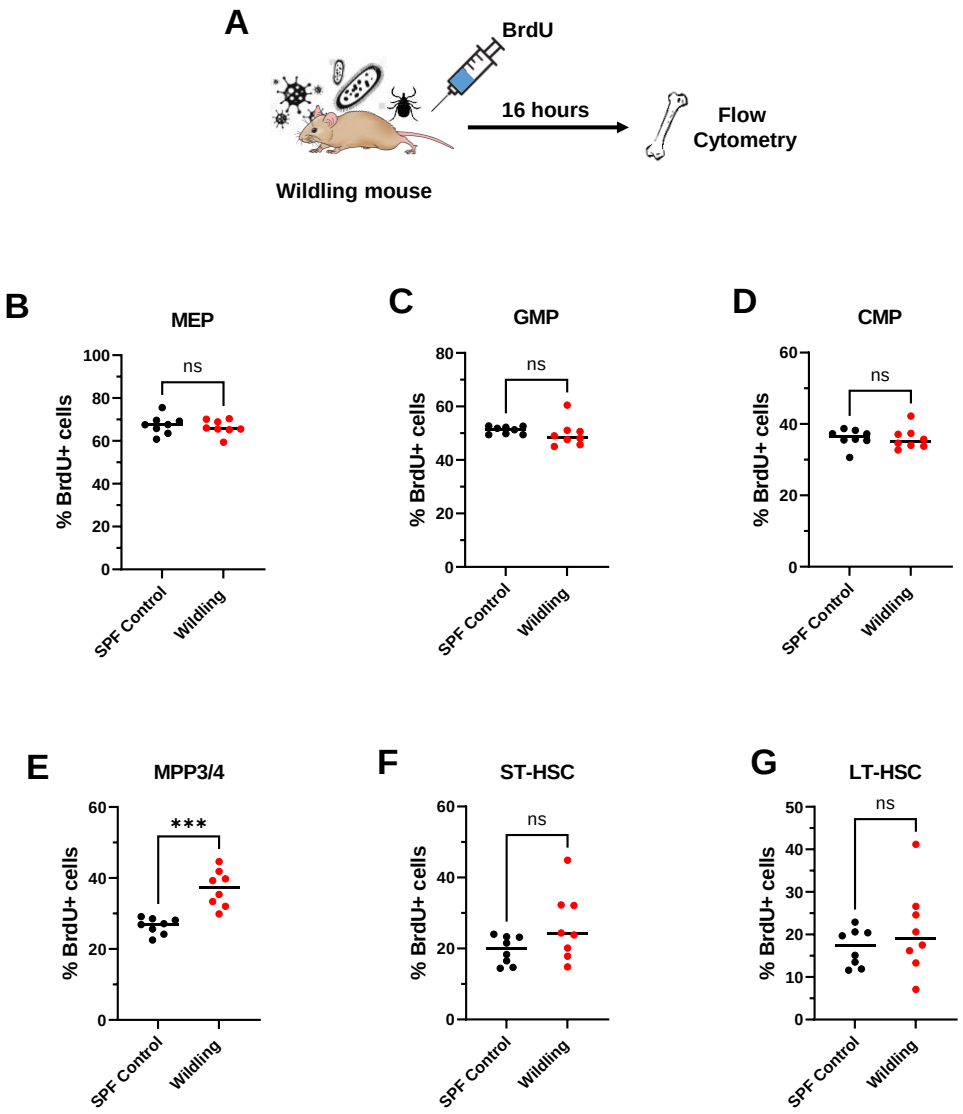


**Figure 5.25: Oligopotent progenitor cells and HSPCs do not show altered proliferation in wildling mice compared to clean SPF mice**

**(A)** Experimental scheme for BrdU incorporation assay to measure the proportion of proliferating cells. **(B)** Percentage of BrdU+ megakaryocyte-erythrocyte progenitor (MEP) cells in the bone marrow of wildling and control mice. **(C)** Percentage of BrdU+ granulocyte-monocyte progenitor (GMP) cells in the bone marrow of wildling and control mice. **(D)** Percentage of BrdU+ common myeloid progenitor (CMP) cells in the bone marrow of wildling and control mice. **(E)** Percentage of BrdU+ MPP3/4 cells in the bone marrow of wildling and control mice. **(F)** Percentage of BrdU+ ST-HSCs in the bone marrow of wildling and control mice. **(G)** Percentage of BrdU+ LT-HSCs in the bone marrow of wildling and control mice

*(n=8 per group; All values are mean±SD; *** represents P<0.001 and ns represents non-significant)*

### 5.4.6. Wildling mice also have reduced spleen size and cell count, similar to long-term co-housed mice

The co-housed mice at the 6 months time-point were found to have some interesting spleen-associated phenotypes due to the infection-response against the long-term colonization of the horizontally transmitted infections, in spite of a lack of strong response in the bone marrow. I next aimed to understand if this is a phenomenon that is also found in the wildling mice. To understand this, I isolated the spleen from the wildling mice used for the co-housing and analyzed the spleen weight and the number of oligopotent progenitors and HSPCs in the spleen.

I observed that there was a significant reduction in the spleen weight and overall white cell count in the spleen of the wildling mice in comparison to the clean control mice **(Figure 5.26A-B)**. There was also a significant reduction in the number of LK cells (Oligopotent progenitors) in the spleen of the wildling mice **(Figure 5.26C)**. There was also a trend observed towards reducing number of LSK cells (HSPCs) in the wildling mice, even though the difference was not statistically significant **(Figure 5.26D)**. All these observations in the spleen of the wildling mice is similar to that what was observed in the 6 months co-housed mice, indicating that the spleen might be an important hematopoietic organ in mice that responds to natural transmission of wild microbiota infections. Since the absolute number of LK cells was elevated in the spleen, I also assessed the proportions of the MEPs, CMPs and GMPs within the LK cell compartment. The wildling mice were observed to have significantly increased proportions of CMPs and GMPs with a concomitant drop in the percentage of MEPs in the spleen **(Figure 5.26E)**. This would mean that there is a push towards increased myelopoiesis in the spleen of the wildling mice, leading to a decrease in absolute numbers of these cells.

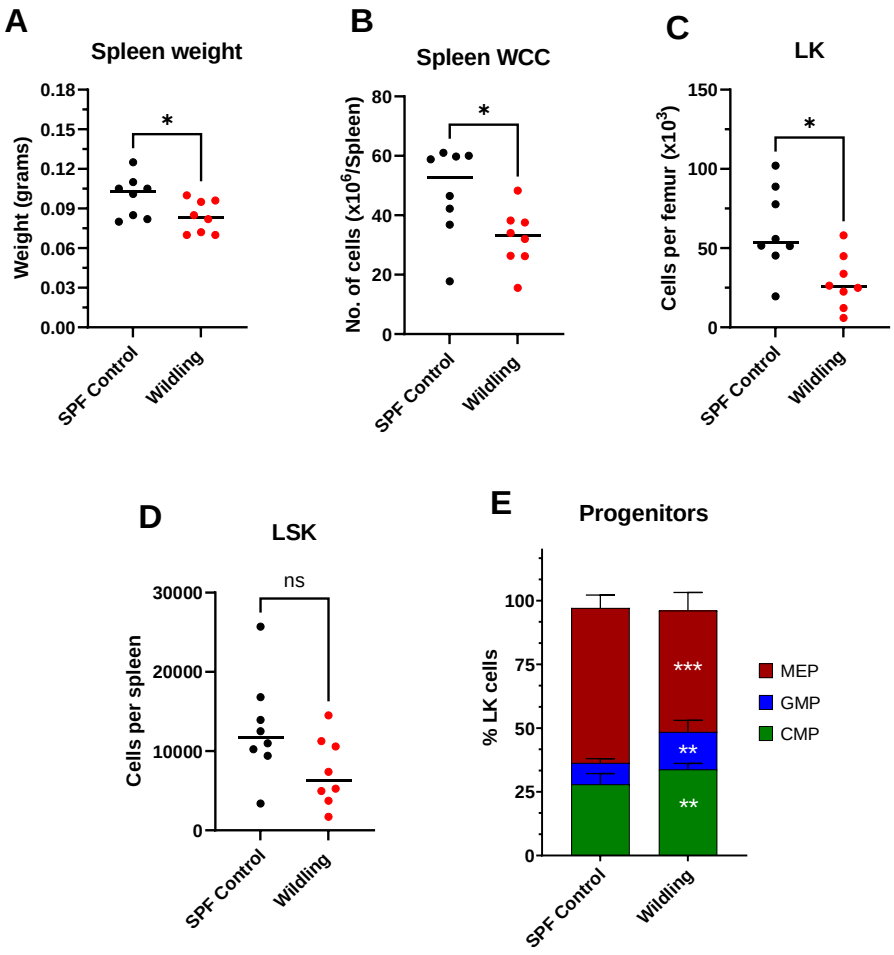


**Figure 5.26: Wildling mice show reduced spleen size and reduced number of oligopotent progenitors and HSPCs**

**(A)** Spleen weight of wilding and control mice. **(B)** Number of white blood cells in the spleen of wilding and control mice. **(C)** Number of LK cells in the spleen of wilding and control mice. **(D)** Number of LSK cells in the spleen of wilding and control mice. **(E)** Proportion of MEPs, CMPs, and GMPs within the LK cell compartment in the spleen of wilding and control mice.

*(n=8 per group; All values are mean±SD; * represents P<0.05 and ns represents non-significant)*

### 5.4.7. Oligopotent progenitors in the spleen of wilding mice have increased proliferation

Since there was an increased proportion of oligopotent progenitors in the spleen of wildling mice, yet their absolute numbers were lower, I assessed whether they have increased proliferation to meet the differentiation-induced reduction in numbers. I therefore performed a BrdU incorporation assay, wherein wildling mice were injected with BrdU (2mg/kg BW) and the spleen was isolated 16 hours post-injection **(Figure 5.27A)**. The spleen LK and LSK cells were analyzed for the proportion of BrdU positive

(proliferating) cells using flow cytometry. It was observed that there was a decrease in the proportion of BrdU positive LSK cells (HSPCs) but an increased proportion of BrdU positive LK cells (oligopotent progenitors) **(Figure 5.27B-C)**. The LK cells are therefore actively proliferating in the spleen of the wildling mice in response to the presence of the infections. Thus, the lower absolute number of LK cells suggests that they are differentiating more than they are proliferating. The LSK cells are proliferating less but might become activated to proliferate once the number of LK cells drops below a certain threshold. Looking at the different LK cell subsets, I also observed that the CMPs, GMPs, and MEPs all have an increased proportion of proliferating BrdU positive cells in the wildling mouse spleen **(Figure 5.27D-F)**. All in all, this data suggests that there is increased proliferation of hematopoietic precursor cells in the spleen of wildling mice and subsequent increased differentiation leading to their overall reduced numbers in the spleen under homeostatic conditions due to the colonized pathogens.

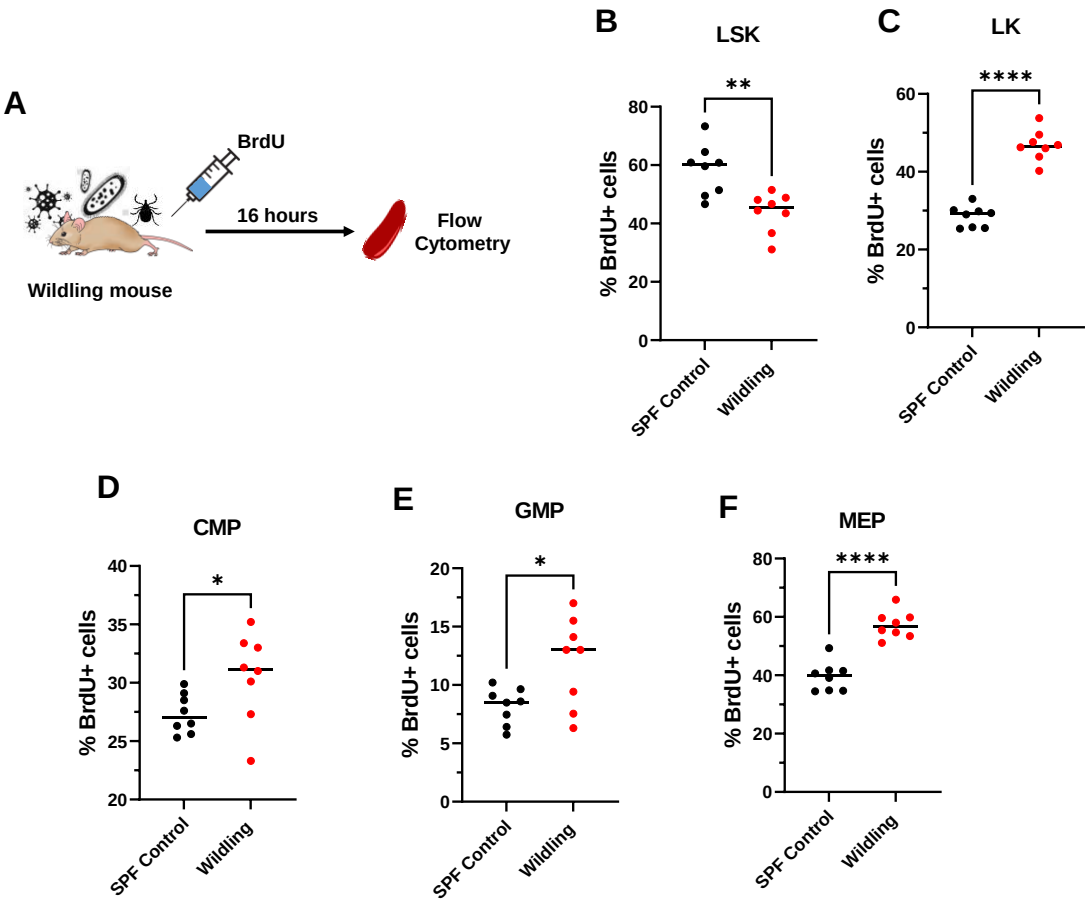


Figure 5.27: Committed progenitors in the spleen of wildling mice have increased proliferation

**(A)** Experimental scheme for BrdU incorporation assay to measure the proportion of proliferating cells in the spleen. **(B)** Percentage of BrdU-positive LSK cells in the spleen of wildling and control mice. **(C)** Percentage of BrdU-positive LK cells in the spleen of wildling and control mice. **(D)** Percentage of BrdU+ common myeloid progenitor (CMP) cells in the spleen of wildling and control mice. **(E)** Percentage of BrdU-positive granulocyte-monocyte progenitor (GMP) cells in the spleen of wildling and control mice. **(F)** Percentage of BrdU-positive megakaryocyte-erythrocyte progenitor (MEP) cells in the spleen of wildling and control mice.
*(n=8 per group; All values are mean±SD; * represents P<0.05, ** represents P<0.01, **** represents P<0.0001 and ns represents non-significant)*

## 5.5. Validating the effects of co-housing on a different DBA2/J laboratory mouse strain

Having studied the co-housing model system as a means to study the effects of natural transmission of infections on the hematopoietic system in C57BL/6J mice, I wanted to assess if similar effects can be observed in other inbred laboratory mouse strains. This would enable us to understand if there are any similarities or differences in terms of infection response across mouse strains. For this, DBA2/J mice were co-housed with a wildling mouse for a period of 3 weeks, similar to that performed with the C57BL6/J mice. The DBA2/J mice were then euthanized 2 months from the start of co-housing to understand the effects of co-housing-mediated acute infections on the blood and bone marrow. DBA2/J mice housed under SPF hygiene conditions were used as control mice.

### 5.5.1. DBA2/J mice do not show altered overall peripheral blood cell counts following co-housing

Contrary to the C57BL6/J mice, the DBA/2J mice do not show any increase in the peripheral blood white cell count after 2 months from the start of co-housing **(Figure 5.28A)**. There were also no significant changes observed in the number of lymphocytes or monocytes **(Figure 5.28B-C)**. There was a modest increase observed in the number of peripheral blood granulocytes **(Figure 5.28D)** indicating a mild infection response, or a previously strong response that subsided at the 2 month's time-point of observation. There was also no differences observed in the red cell count in the co-housed DBA2/J mice compared to the clean control mice, but a drop in platelet count was observed in the co-housed mice **(Figure 5.28E-F)**. Overall, the observations

indicate a very mild response to transfer of infections through co-housing compared to the C57BL6/J mice that were co-housed for 2 months.

To further understand the lack of altered peripheral blood cell counts in the co-housed mice, I analyzed the proportion of the different CD4+ and CD8+ T-cell subsets. I observed that there was a significantly higher proportions of naïve CD4+ and CD8+ T-cells in the peripheral blood of co-housed mice compared to control mice **(Figure 5.28G-H)**. There was a concomitant reduction in the percentage of active CD4+ T-cells and memory CD8+ T-cells. These observations are in striking contrast to that observed in the co-housed C57BL/6J mice at 2 months after co-housing. This might be due to a delayed infection response in this mouse strain and the kinetics of change in peripheral blood counts following infection transmission might be different between difference inbred mouse strains.

### 5.5.2. Co-housed DBA2/J mice show no significant changes in the number of oligopotent progenitors or HSPCs in the bone marrow

The bone marrow of the DBA2/J mice was studied 2 months after the start of co-housing to assess and compare the acute effects of co-housing mediated infections on the bone marrow progenitor cells and HSCs. There was no changes observed in the absolute number of LK cells (oligopotent progenitors) in the bone marrow of co-housed mice **(Figure 5.29A)**. There was however a trend towards a modest decrease in the number of GMPs and an increase in the CLPs in the bone marrow **(Figure 5.29B-C)**. However the differences were not statistically significant, meaning that I could not draw firm conclusions about this data. There were also no changes found in the number of LSK cells (HSPCs) in the co-housed mice **(Figure 5.29D)**. A small increase was observed in the number of myeloid primed MPP3 cells **(Figure 5.29E)**, which might be a consequence of the small drop in GMP numbers. The number of the LT-HSCs was not found to be altered due to the co-housing **(Figure 5.29F)**, similar to that of both the co-housed mice and wildling mice. Taken together, the peripheral blood and the bone marrow analysis revealed pronounced differences in how the two different mouse strains respond to the acute infections acquired through co-housing.

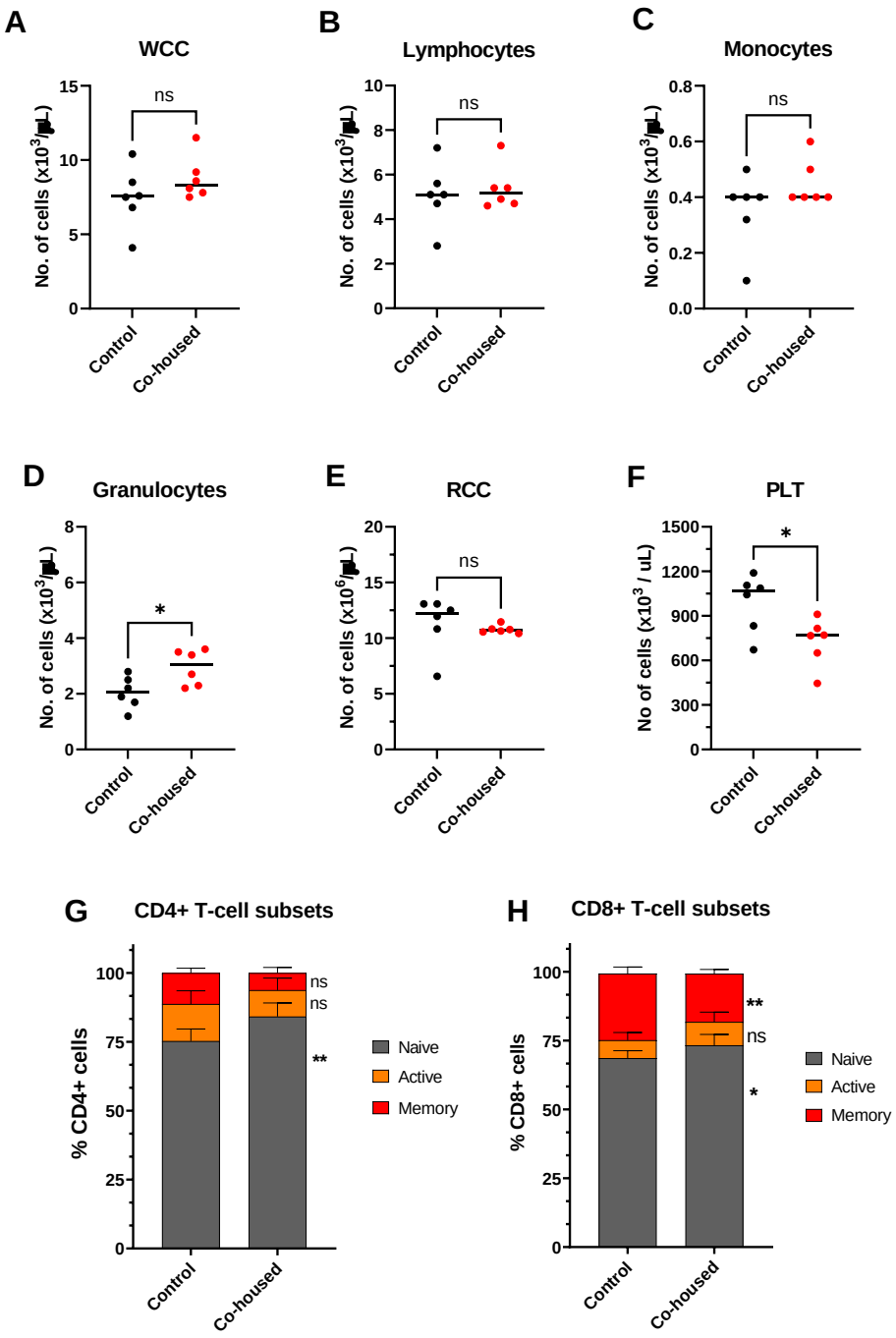


**Figure 5.28: Co-housed DBA2/J mice do not show altered peripheral blood counts but there was an increased proportion of naïve T-cells**

**(A)** Peripheral blood white blood cell count of DBA/2J mice at 2 months after co-housing. **(B)** Peripheral blood lymphocyte count of DBA/2J mice at 2 months after co-housing. **(C)** Peripheral blood monocyte count of DBA/2J mice at 2 months of co-housing. **(D)** Peripheral blood granulocyte counts of DBA/2J mice at 2 months of co-housing. **(E)** Peripheral blood red blood cell count of DBA/2J mice at 2 months of co-housing. **(F)** Peripheral blood platelet count of DBA/2J mice at 2 months of co-housing. **(G)** Proportion of different CD4+ T-cell subsets in the peripheral blood of DBA2/J mice at 2 months of co-housing. **(H)** Proportion of different CD8+ T-cell subsets in the peripheral blood of DBA2/J mice at 2 months of co-housing.

*(n=6 per group; All values are mean±SD; * represents P<0.05, ** represents P<0.01 and ns represents non-significant)*

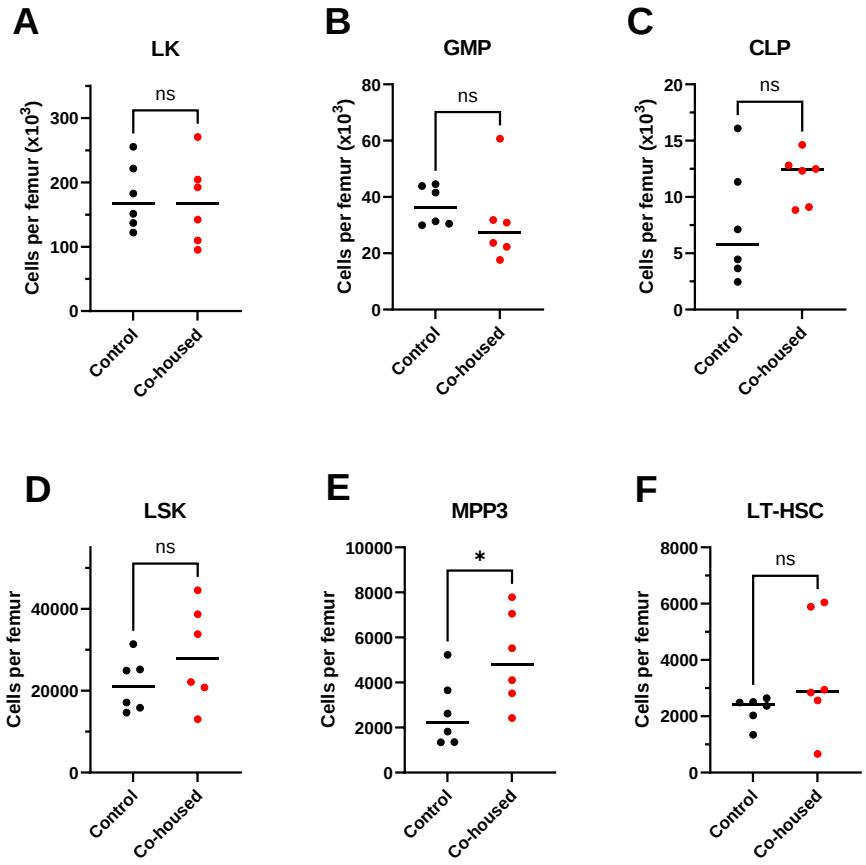


**Figure 5.29: Co-housed DBA2/J mice show no significant changes in the number of oligopotent progenitors or HSPCs in the bone marrow**

**(A)** Number of LK cells in the bone marrow of DBA/2J mice at 2 months after co-housing. **(B)** Number of granulocyte-monocyte progenitor (GMP) cells in the bone marrow of DBA/2J mice at 2 months after co-housing. **(C)** Number of common lymphoid progenitor (CLP) cells in the bone marrow of DBA/2J mice at 2 months of co-housing. **(D)** Number of LSK cells in the bone marrow of DBA/2J mice at 2 months of co-housing. **(E)** Number of MPP3 cells in the bone marrow of DBA/2J mice at 2 months of co-housing. **(F)** Number of LT-HSCs in the bone marrow of DBA/2J mice at 2 months of co-housing.

*(n=6 per group; All values are mean±SD; * represents P<0.05 and ns represents non-significant)*

## 5.6. Using the co-housing model system to study how infections can affect mice carrying mutations associated with clonal hematopoiesis

Throughout the life of an individual, hematopoietic stem cells are known to acquire somatic mutations without any apparent signs of a hematological disorder.

These observations indicate that HSCs that acquire certain mutations hold a functional or proliferative advantage, facilitating thier accumulation in individuals without directly causing a disease phenotype. This clonal expansion of a specific subset of HSCs during aging is referred to as clonal hematopoiesis of indeterminate potential (CHIP). These age-related clonal mosaicisms in healthy individuals have been predicted to be a risk factor for later development of hematopoietic neoplasms (215). The most frequent mutations in individuals that exhibit clonal hematopoiesis have been observed to be mutations in epigenetic regulators such as TET2 and DNMT3A (101). Furthermore, inflammation and infection-induced proliferation of HSCs harboring these CHIP mutations has been shown to accelerate clonal hematopoiesis in mouse models (112,113). Hence I wanted to assess how the co-housing mediated colonization of wild microbiota in my study affects hematopoiesis with mice harboring CHIP mutations in HSCs.

Previously published Cre-recombinase inducible Tet2 knockout mice (216) and Dnmt3a-R878H mutant mice (217) were crossed with Tamoxifen-inducible SclCre mice which express the Cre recombinase in the HSPCs. Bone marrow from the resultant CD45.1 Tet2, Dnmt3a or SclCre control mice were transplanted along with CD45.1/2 wildtype competitor mouse bone marrow in a 1:1 ratio into lethally irradiated CD45.2 recipient mice. The resultant chimeric mice are referred to as Tet2, D3a or Cre mice in all figures. 4 months after transplant, the chimeric mice were administered with Tamoxifen to activate the Cre recombinase and the subsequent induction of Tet2 knockout and mutant Dnmt3a expression. The mutant mice and SclCre control mice were then co-housed with a wildling mouse in the same cage for a period of 3 weeks to allow the natural transmission of infections. The transplanted mice were then bled once every 4 weeks for a period of 16 weeks. At the end of 16 weeks the mice were euthanized and the bone marrow was analyzed. Non co-housed Tet2, D3a and Cre mice housed under SPF condition were used as clean control mice.

All flow cytometry analysis in this part of the thesis was performed by a master student (Dhyani Shah) as part of her master degree dissertation, under my direct supervision.

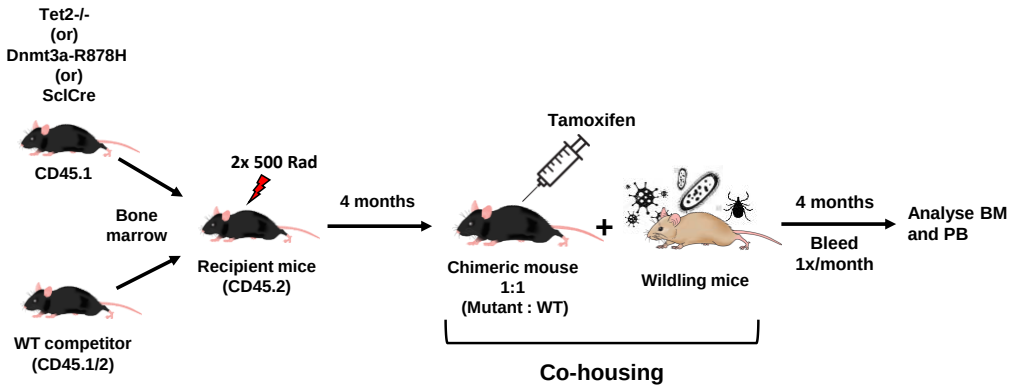


Figure 5.30: Experimental scheme to study the effect of co-housing on mice with mutations associated with clonal hematopoiesis

### 5.6.1. Tet2 knockout mice show modest increase in peripheral blood donor chimerism but is not co-housing dependent

Firstly, I assessed the overall white cell count in the peripheral blood of the Tet2, D3a and Cre mice over a period of 16 weeks. It was observed that the Tet2, D3a and Cre mice that were co-housed had a trend towards elevated white cell count at 4 weeks of co-housing, suggesting an immune response towards the infections transmitted **(Figure 31A-B)**. However, the elevated blood count was not sustained over the subsequent blood analysis time-points. Also, the increase in white cell count was not as high as that observed in wildtype mice that were co-housed to validate the model. This might be due to the transplanted mice responding differently to infections compared to primary mice.

Having validated the slight increase in white cell count indicating the presence of a co-housing mediated infection response, I analyzed the overall peripheral blood CD45.1 donor chimerism and across the myeloid, B-cell and T-cell lineages through flow cytometry **(Figure 5.32A)**. The total peripheral blood donor chimerism before inducing the mutation with Tamoxifen was already observed to be lower than the transplanted 50% value for all the experimental groups **(Figure 5.32B)**. This was the lowest for the D3a transplanted cells, indicating some form of bone marrow engraftment defect even before the induction of the mutation and co-housing. Also, there were small differences in the percentage engraftment across the different mice in the same experimental group.

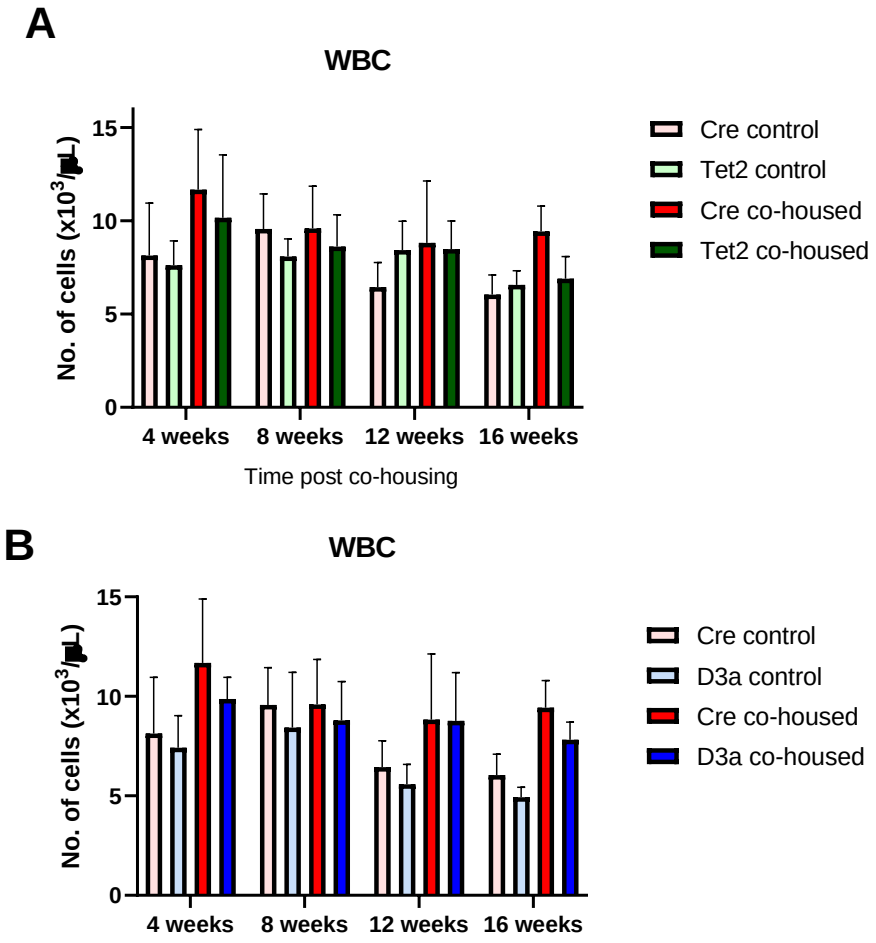


**Figure 5.31: Both control and co-housed mice show slightly elevated peripheral blood white cell count at 4 weeks after co-housing**

**(A)** Peripheral blood white blood cell (WBC) count Tet2 and Cre mice at different time-points from start of co-housing. **(B)** Peripheral blood white blood cell (WBC) count of D3a and Cre mice at different time-points from start of co-housing.

*(n=5-7 per group; All values are mean±SD)*

Since there were differences in initial donor engraftment percentages across the different mice within the same experimental group, for all subsequent timepoints of blood sampling the donor chimerism percentages were normalized to their pre-co-housing values. There was a trend towards a higher total peripheral blood chimerism at 16 weeks in the Tet2 mice. However, this increase was similar in both the co-housed and the clean control groups **(Figure 5.33A)**. This would corroborate previous observations in the literature that report that Tet2 knockout leads to a competitive advantage for the hematopoietic cells to expand (216). The lack of differential response to co-housing might be due to the shorter analysis period from the start of co-housing.

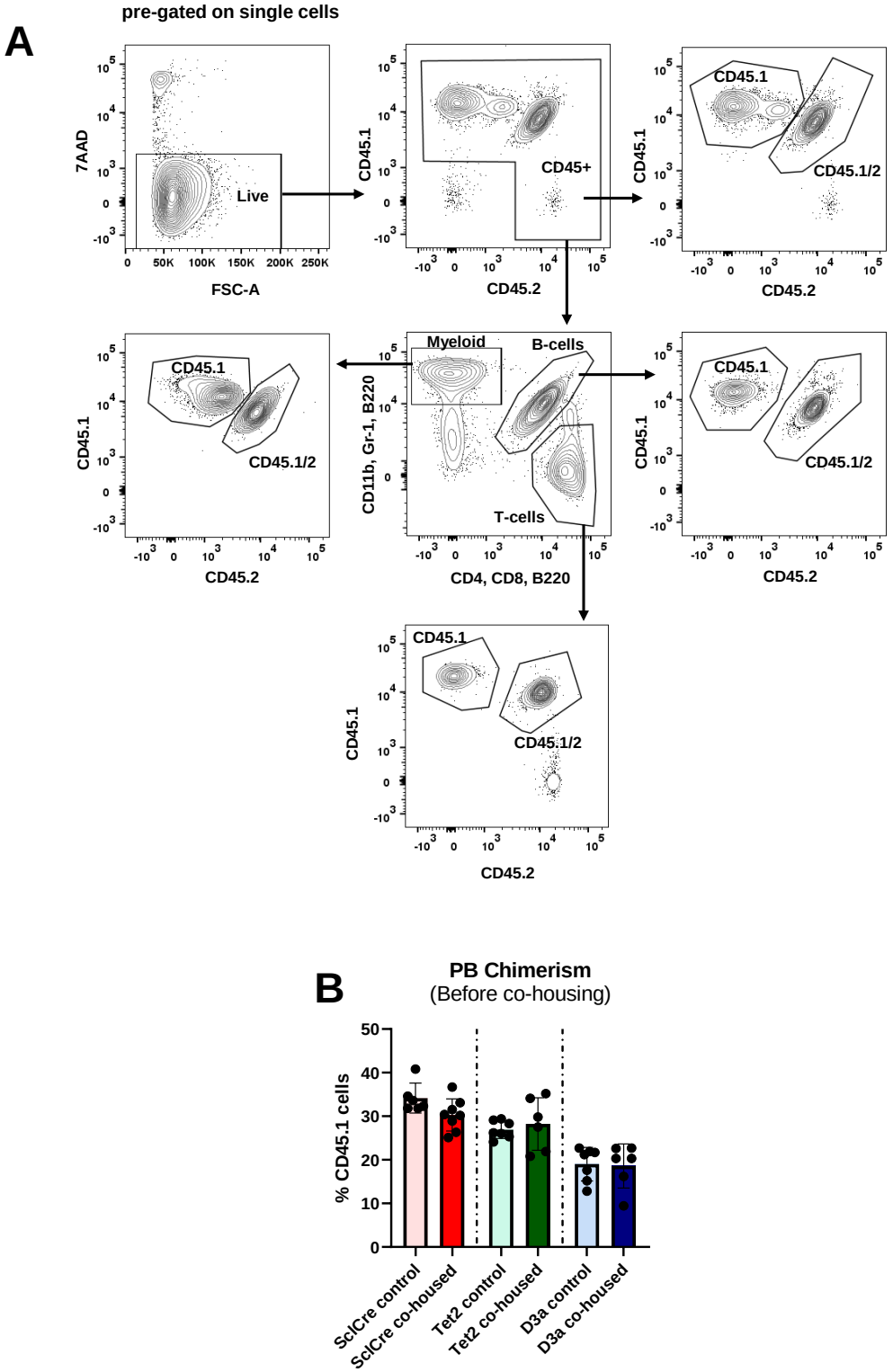


**Figure 5.32: Pre-co-housing peripheral blood donor chimerism of transplanted mice**
**(A)** Flow cytometry scheme to assess the percentage donor chimerism in myeloid, T-cells and B-cells in the peripheral blood. **(B)** Total peripheral blood CD45.1 donor chimerism before induction of the mutation with Tamoxifen and co-housing.
*(n=5-7 per group; All values are mean±SD)*

Longer term assessment of the peripheral blood might be required to more comprehensively interrogate this phenomenon. Furthermore, looking at the donor chimerism changes within the myeloid and T-cells in the peripheral blood, it was observed that the small increase in chimerism of Tet2 cells was specific to the myeloid and not the T-cells at 16 weeks **(Figure 5.33B-C)**. This would indicate that the Tet2 knockout leads to a biased differentiation towards the myeloid lineage. However, again the differences are for both co-housed and control Tet2 mice and is therefore independent of co-housing.

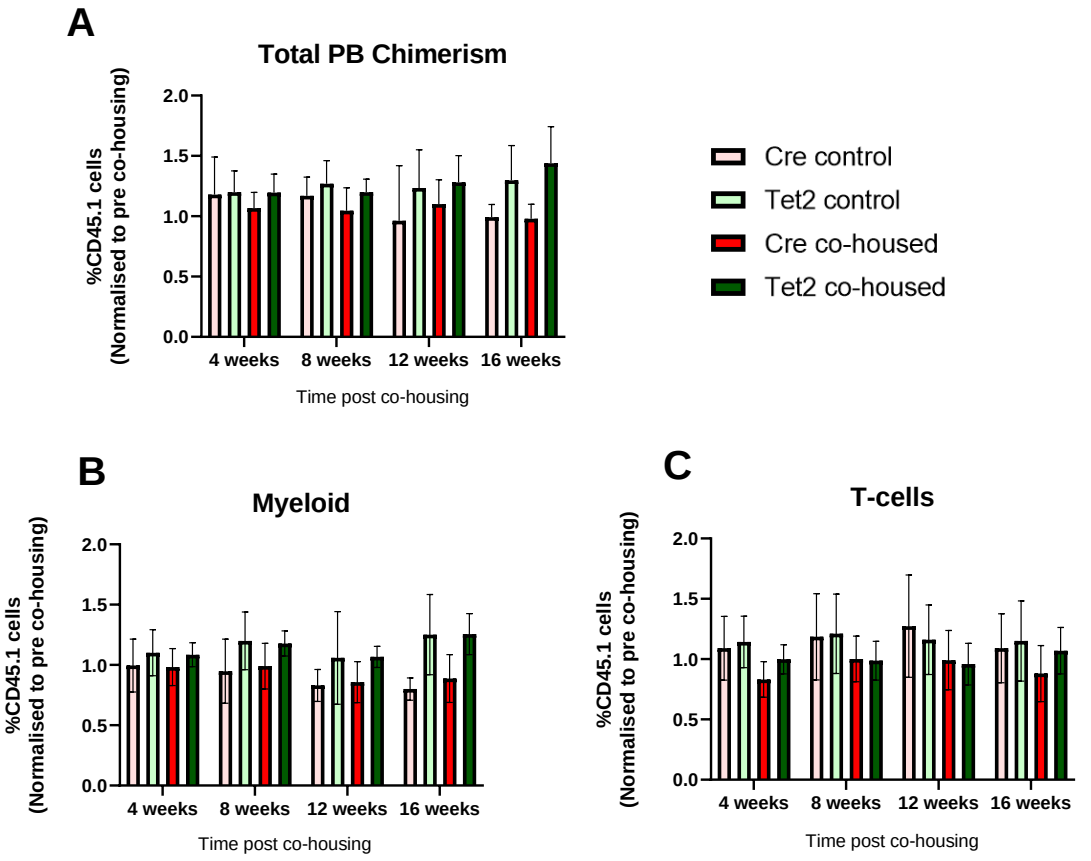


**Figure 5.33: Tet2 deficient cells show slightly increased peripheral blood contribution but is not co-housing dependent**

**(A)** Total peripheral blood CD45.1 donor chimerism in Tet2 and Cre mice at different time-points from the start of co-housing. **(B)** Peripheral blood CD45.1 donor derived myeloid cell chimerism in Tet2 and Cre mice at different time-points from the start of co-housing. **(C)** Peripheral blood CD45.1 donor derived T-cell chimerism in Tet2 and Cre mice at different time-points from the start of co-housing.

*(n=5-7 per group; All values are mean±SD)*

The Dnmt3a mutant cells on the other hand showed a progressive decrease in peripheral blood donor chimerism **(Figure 5.34A)**, reiterating the engraftment defect already observed during the pre-tamoxifen chimerism percentage. Hence, it is not feasible to compare the differences between the Cre and the D3a mice. However, comparing the overall donor chimerism between the co-housed and the control, Again, a longer term assessment of donor chimerism may be more informative since in previous studies where high dose pathogens were directly injected into Dnmt3a-deficient mice, the expansion of peripheral blood mature cell chimerism was only observed after a year of chronic infection (113). There was also no observed differences in the donor chimerism percentage among the myeloid and T-cells **(Figure 5.33B-C)**, unlike what we saw in Tet2 mice.

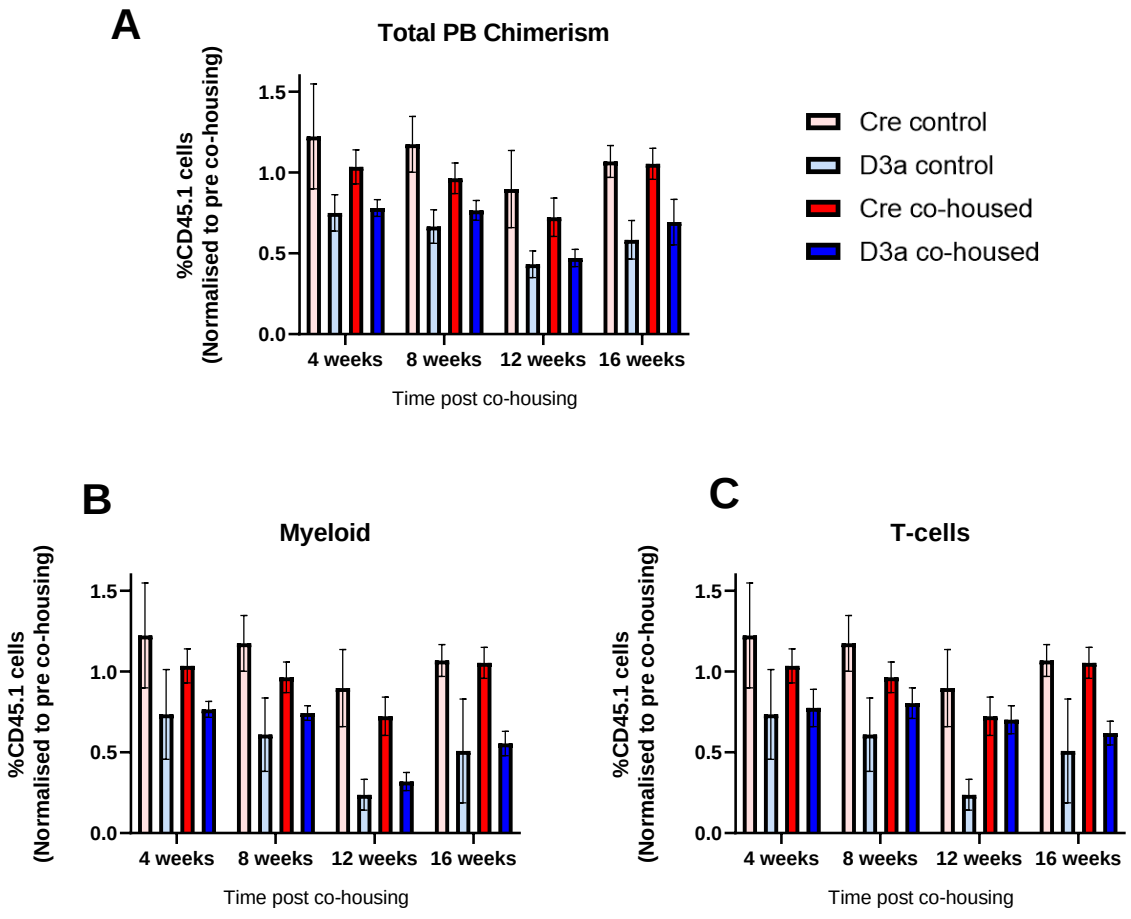


**Figure 5.34: Dnmt3a mutant cells showed reduced overall initial engraftment and no increase in peripheral blood contribution**

**(A)** Total peripheral blood CD45.1 donor chimerism in D3a and Cre mice at different time-points from the start of co-housing. **(B)** Peripheral blood CD45.1 donor derived myeloid cell chimerism in D3a and Cre mice at different time-points from the start of co-housing. **(C)** Peripheral blood CD45.1

donor derived T-cell chimerism in D3a and Cre mice at different time-points from the start of co-housing.
*(n=5-7 per group; All values are mean±SD)*

### 5.6.2. Tet2 knockout and Dnmt3a mutant mice do not show any differences in the frequency of HSPCs in the bone marrow

Since Tet2 and Dnmt3a CHIP-associated mutations lead to an expansion of HSPCs in the bone marrow, I analyzed the bone marrow of the Tet2 and D3a mice to assess any changes in their frequencies with or without co-housing. Compared to their respective non-co-housed control mice, neither Tet2 nor D3a co-housed mice showed any changes in the frequency of CD45.1 donor LSK cells (HSPCs) or specifically the MPP3/4, ST- or LT-HSCs in the bone marrow at 16 weeks of co-housing **(Figure 5.35A-D)**. However the non-housed control Tet2 and D3a mice also did not exhibit any differences compared to the Cre control mice. This would indicate that at the 16 week timepoint, there is no competitive advantage or expansion of HSPCs in the mutant mice, and there is no additional effect of co-housing on HSPC competitive advantage. Hence a longer time post induction of mutation might be required. This is however surprising for the Tet2 mice, as it has been previously shown that Tet2 knockout alone shows very rapid competitive advantage over wildtype bone marrow in a competitive transplant setting (216). The recombination efficiencies post-tamoxifen treatment must also be assessed here to understand any potential limitation with using the SclCre system here.

In conclusion, co-housing mediated transmission and colonization of infections does not appear to have any differential effects on hematopoiesis in mice carrying CHIP mutations. Whether this lack of differential response is due to the mutation or the mildness of co-housing mediated infection response is to be studied further.

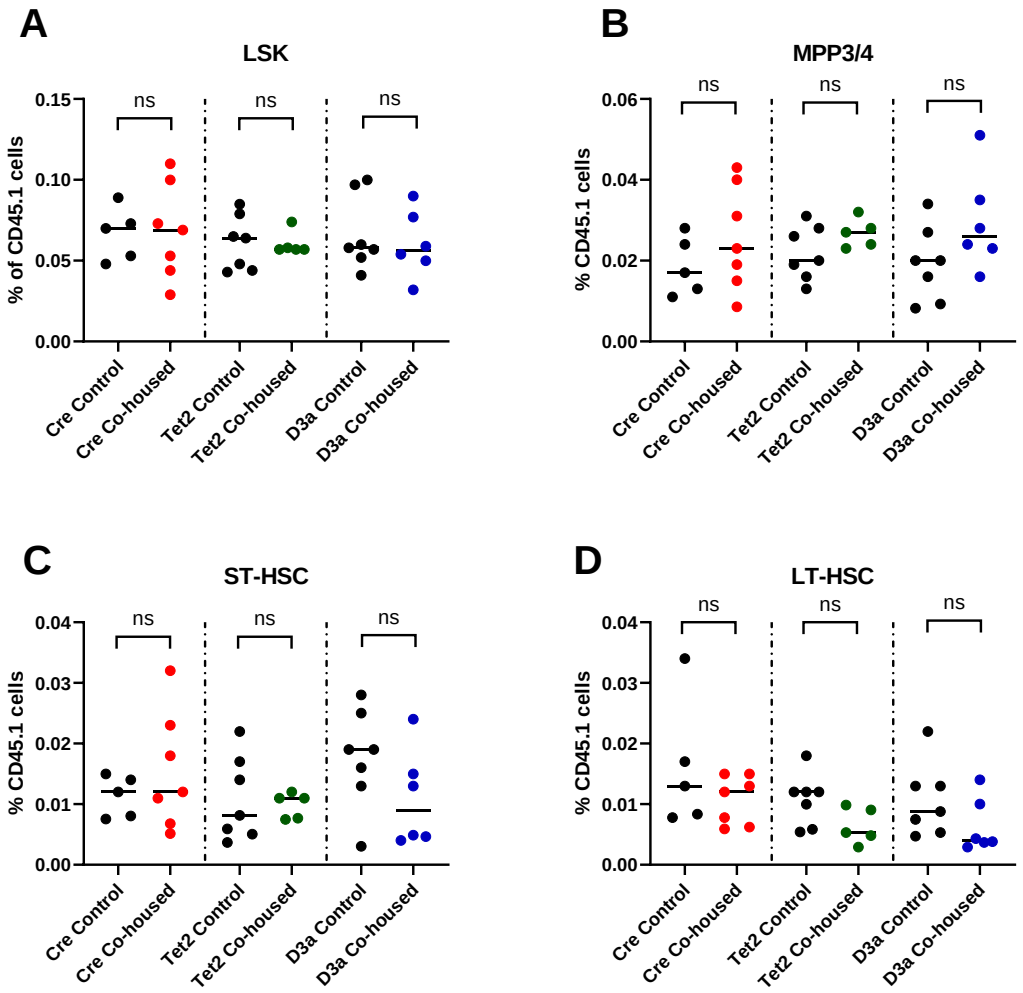


**Figure 5.35: Mice with mutations associated with clonal hematopoiesis mutations do not show altered HSPC frequencies in the bone marrow**

**(A)** Frequency of LSK cells within the CD45.1 donor cells in the bone marrow of Cre, Tet2 and D3a mice at 4 months after co-housing. **(B)** Frequency of MPP3/4 cells within the CD45.1 donor cells in the bone marrow of Cre, Tet2 and D3a mice at 4 months after co-housing. **(C)** Frequency of ST-HSCs within the CD45.1 donor cells in the bone marrow of Cre, Tet2 and D3a mice at 4 months after co-housing. **(D)** Frequency of LT-HSCs within the CD45.1 donor cells in the bone marrow of Cre, Tet2 and D3a mice at 4 months after co-housing.

*(n=5-7 per group; All values are mean±SD; ns represents non-significant)*

# 6 Discussion - II

## Co-housing model to study the effect of infections on hematopoiesis and HSC biology

The production of diverse mature cells present in the peripheral blood is of paramount importance to the survival of any organism. This process of blood cell production, under homeostatic conditions, from the hematopoietic stem cells (HSCs) that reside in the bone marrow is referred to as steady-state hematopoiesis. This exemplifies the HSCs as some of the most important cells in an individual and its functional preservation as a key factor for survival. Under steady-state, these important HSCs exist in a dormant state in the bone marrow and rarely proliferate, in order to preserve their functional fitness (25). However, under conditions of stress such as blood loss, chemotherapy and infections, these HSCs are driven into active proliferation to meet the demands of increased mature blood cell production (28). This increased proliferation has been shown to come at the cost of reduction in their functional potency to efficiently repopulate the hematopoietic system.

Throughout the course of an individual's lifetime, the person experiences multiple infections and the corresponding need for immune response that goes along with it. This makes infection-stress as one of the most common mediators of stress-induced hematopoiesis and the potential negative mediator of HSC fitness. Over time, the effects of different infections on hematopoiesis and the different bone marrow cells can accumulate, potentially leading to hematological disorders with age. This has warranted the plethora of studies to understand how infections mediate their effect on hematopoiesis and how these infections could elicit their negative effects on HSC biology. To this end, different mouse models of infections have been studied over the years. Some of these include injecting mice with pI:pC (viral dsRNA mimetic), directly injecting mice with highly pathogenic microorganisms such as *Mycobacterium avium* and murine cytomegalovirus (mCMV) or using mosquitoes as vectors to infect mice

with *Plasmodium berghei* (28,30,76,77). Among others, these models have been specifically used to assess the effects of acute and chronic infections on HSCs and were found to have detrimental effects on HSC function. However, the major caveat with all of these models is that they either use a compound that only mimics an infection (pI:pC) or use injections of non-physiological high-dose purified pathogens into mice. This does not recapitulate a natural process of infection initiation or response that normally occurs in humans who acquire less severe and common infections on a day to day basis.

A more recent approach of co-housing laboratory mice with infectious mice from a pet-store that carry a multitude of infectious microbiota has shown to elicit a more natural transmission of pathogens and the associated peripheral immune response (84). However, this study only assessed the effects of co-housing mediated infections on peripheral immunity and not on the changes in bone marrow hematopoiesis. Meanwhile, studies by Rosshart *et al*., has led to the generation of the so-called wildling mice that carry a range of wild-mouse microbiota and infections, and are based on a C57BL/6 background, thereby also offering the genetic tractability of a laboratory mouse (89). Hence in this part of the doctoral thesis, I assessed the effects of co-housing mediated infections on the different layers of the hematopoietic hierarchy, using these wildling mice. Furthermore, all infection models use laboratory mice that are housed under clean specific-pathogen-free (SPF) hygiene conditions before the infections occur. These mice are hence immunologically naïve before the infections. This is in contrast to humans who face multiple pathogens right from birth. Having the wildling mice, hence enabled me to also study how acquiring the same infections from birth differs in hematopoietic response to that of mice that acquire the infections only in adulthood during co-housing.

## 6.1. Co-housing clean mice housed under SPF conditions with dirty wildling mice elicits acute and chronic immune response, that validates the model

Firstly, a good model system to study infections and microbial colonization would be one where there is a natural transmission of pathogens. This is what was established previously by the work of Beura *et al* using pet-store mice to perform co-housing experiments. They found a range of infections in the pet-store mice that were

sampled and also observed good transmission of the infections into the co-housed laboratory mice (84). I was able to recapitulate this using the wildling mice in my work, as observed by the efficient natural transmission of a multitude of infections into the co-housed mice. However, the use of pet-store mouse for co-housing was accompanied by a 20% rate of death in the co-housed mice due to infection-related morbidity. However, this was not the case in any of the experiments performed as part of this thesis using the wildling mice. This would indicate a milder infection and colonization of pathogens using the wildling mice. This was also apparent in the range of infections observed in the pet-store mice versus that observed in the wildling mice (84,89). This could be seen as an advantage of using wildling mice for co-housing, as the pathogenicity of the infections transmitted is more in line with those encountered by humans during daily life.

Secondly, a good model system of infections should elicit an active immune response in the mice. This was also observed when clean SPF mice were co-housed with wildling mice, mediating an acute increase in the number of peripheral blood mature cells 2 months from the start of co-housing. The increase in peripheral blood counts was observed across all the immune cells including granulocytes, monocytes, B-cells and T-cells. There was also an increase in the proportion of active CD4+ T-cells in the peripheral blood of the co-housed mice. This observation is in line with what was observed in the co-housing experiments with pet-store mice by Beura *et al* (84). But it has to be noted here that the change in proportion of active CD4+ T-cells was higher in the study performed using pet-store mice, probably owing to the increased pathogenicity of the infections present in the pet-store mice compared to the wildling mice. However, the maximum duration post initiation of co-housing was only 52 days (3 months) in the study involving pet-store mice. In my study I extended this duration to 6 months of co-housing to assess how chronic colonization with infections affects hematopoiesis. The mice co-housed with wildling mice here were observed to have a sustained high number of white blood cells in the peripheral blood including both innate and adaptive immune cells. The microbiology analysis also showed that the co-housed mice still had live pathogens in the various organs analyzed at 6 months of co-housing. This strongly suggests that the mice have an ongoing active immune response against the multitude of infections acquired during the co-housing phase, even at 6 months after the start of co-housing. These observations hence validates the co-housing model proposed here as part of this thesis to be suitable for studying both acute and chronic effects of infections on the hematopoietic system.

## 6.2. Comparing the effects of horizontal and vertical transmission of infections on the hematopoietic system

In humans, it is speculated that the microbial colonization starts as early as a neonate through exposure to microbes in the mother's amniotic fluid and birth canal, followed by exposure to those in the mother's milk and those present in the immediate environment following birth (218,219). The composition of microbiota has also been linked to affect immune response to subsequent infections and sepsis in neonatal mice (220). This puts emphasis on the timing of exposure to microbes as a neonate versus that as an adult, and their subsequent effects on hematopoiesis. In line with this, the co-housed mice analyzed at 6 months after initiation of co-housing, represent long-term horizontal transmission of infections from one mouse to another. On the other hand, the wildling mice having acquired the infections from birth represents a case of vertical transmission of infections. The differential effects that these two modes of transmission of infections can have on hematopoiesis and HSCs has not been previously studied. The access to these two scenarios with the co-housed and the wildling mice enabled me to explore this aspect of hematopoiesis further in this thesis. Hence I also analyzed the wildling mice that were used for the co-housing along with the co-housed mice. Mice in both these experimental groups were of similar age and represented a similar infection spectrum. The difference was only in terms of timing of exposure to the wild microbiota infections.

### 6.2.1. Co-housed mice differ from wildling mice in terms of changes to the mature peripheral blood cells

As is expected during an active immune response to infections, the co-housed mice showed significantly elevated number of peripheral blood mature cells such as granulocytes, monocytes, T-cells and B-cells, even at 6 months from start of co-housing. The presence of active immune response for such a long duration against the colonized infections is intriguing since in previous models of chronic infections such as *Mycobacterium avium* and mCMV, the mice had to be regularly injected with the pathogens to maintain an active immune response (30,76). Hence, even though the wild microbiota transmitted to the co-housed mice are not highly pathogenic, they are

still strongly colonized and constantly eliciting an immune response in the mice. However, the persistently elevated immune cells in the peripheral blood could also mean that the different microbes transmitted induce an immune response at different points of time after co-housing. On the other hand, the previous models of injecting pathogens into mice involved a single pathogen. These pathogens were probably synchronized in their infectious phases, during culture and subsequent injection into mice.

The peripheral blood observations in the wildling mice were however in striking contrast to that observed in the co-housed mice. The wildling mice showed no differences in the peripheral blood cell counts compared to clean control mice. However, the wildling mice were still observed to have live microbes present in different organs, as was observed in the microbiological analysis of these mice. This would have warranted an active immune response towards these microbes as observed in the co-housed mice that were of a similar age. Hence, the lack of elevated blood counts would indicate that having the wild microbiota and infections right from birth, somehow normalizes the peripheral blood counts in the adult wildling mice to the same steady-state values of immunologically naïve SPF mice. It could also be that the turnover rate of mature blood cells is so high that the absolute number of these cells does not appear to be elevated at a given time-point. Another potential reason for this could also be that the immune response just normalized over time due to the longer duration of presence of the microbes as opposed to the timing of acquisition of the infections. To understand this, peripheral blood of slightly younger wildling mice could be analyzed to assess if the immune response just gradually reduces over time once the required threshold of humoral immune response is in place to keep the infections in check. Furthermore, the microbial load could be assessed if the load of different microbes is the same between co-housed mice and wildling mice at similar ages. Any differences in the gut microbiome between the wildling mice and the co-housed mice could also have differential effects in immune response to infections. Even though the co-housed and wildling mice should have similar gut microbiome due to co-housing, there could be minor differences. Even subtle changes in the gut microbiome have been previously shown to have impact on immune responses and the presence of mature immune cells in peripheral sites (221,222). The level of myelopoiesis and granulopoiesis in the bone marrow can also be a consequence of differences in gut microbial composition (223,224). Fecal pellets from the wildling mice and co-housed

mice were collected but has not yet been analyzed for gut microbiome composition. This would give an answer to the above mentioned speculation.

In conclusion, the wildling mice and the long-term co-housed mice have striking differences in the peripheral blood cell counts, but the exact reason for this difference is not yet clear.

### 6.2.2. Both horizontal and vertical transmission of infections lead to altered hematopoiesis in the spleen but not similar to other infection models

Under certain conditions during development and stress, hematopoiesis has been shown to occur at sites other than the bone marrow (225). This is referred to as extramedullary hematopoiesis. Among other sites, spleen is a major organ where this phenomenon of extramedullary hematopoiesis has been shown to occur during infections (209–212). This involves mobilization of HSPCs from the bone marrow into the spleen and their subsequent expansion in number. To understand if this also occurs during co-housing and in the wildling mice, the spleen of these mice were analyzed.

Both the long-term co-housed mice and the wildling mice were observed to have altered HSPC numbers in the spleen. However, the change observed was opposite to that which normally occurs in case of extramedullary hematopoiesis. The spleen HSPC and oligopotent progenitor numbers were found to have decreased in the co-housed and the wildling mice, as opposed to an expected expansion in number. This could be due to a potential switch of mature blood cell production from the spleen instead of the bone marrow, in conjunction with a lack of mobilization of HSCs from the bone marrow to the spleen. This increased differentiation and a lack of mobilization from bone marrow might explain the reduction in their number, in spite of the increased proliferation of spleen HSPCs and oligopotent progenitors in wilding mice observed in the BrdU incorporation assay. This theory would be supported by the observation that in the wildling mice there is an increased proportion of GMPs and CMPs with a concomitant decrease in MEPs within the LK cell compartment, meaning that there is increased granulopoiesis in the spleen. Moreover, the observed decline in spleen HSPCs was consistent across both the co-housed mice and the wildlings suggesting a common phenomenon that can occur with both horizontal and vertical transmission of infections. This indicates that the phenotype is transmissible. Based on published

literature, the involvement of spleen in response to infections appears to be dependent on the type of infection in mice. For example, while *Mycobacterium avium* and *Plasmodium berghei* infections in mice has been shown to induce the expansion of HSPCs in the spleen, *Ehrlichia chaffeensis* infections do not induce this phenotype in the infected mice (40,226,227). Hence, this specific effect on the spleen hematopoiesis could be dependent on a very particular microbe that is present in the wildling mice and is transmitted to the co-housed mice. Further correlative assessment of the presence or absence of a particular infection and the spleen cell count might be required to ascertain this hypothesis.

### 6.2.3. Natural transmission of wild microbiota and infections does not alter the number of progenitors or HSCs in the bone marrow

Every single model studying systemic infections in mice has shown that infections lead to altered bone marrow composition with an expansion of the LSK (HSPC) compartment accompanied by the specific proliferation of the HSCs (53,76,228–230). This is considered a classical hallmark of infection response in mouse models and is totally plausible for these models since systemic infections demand for increased production of mature blood cells to clear the infections. This would in turn require expansion and differentiation of the various progenitor cells in the bone marrow. Also different bacterial and viral infections are known to cause massive production of cytokines including various pro-inflammatory cytokines such as interferons, interleukins, etc., which are known to drive HSCs into proliferation (27,57,231). These mechanisms have been attributed to the heightened bone marrow response in various models of infection.

However, neither the co-housed mice nor the wildling mice showed an increase in the composition of any of the bone marrow oligopotent progenitor cells or HSPCs. Also, there was no changes in HSC proliferation observed in the co-housed mice and the wildling mice. This would mean that the natural colonization of wild microbiota does not elicit such a strong bone marrow response as opposed to more pathogenic systemic infections that lead to a cytokine storm. Analyzing the level of different pro-inflammatory and anti-microbial cytokines in the serum and bone marrow of these mice would give a better understanding if the infection response is indeed milder with natural microbial transmission with the wildling mice. Analysis of the transcriptome

of the peripheral blood mononuclear cells in mice co-housed with pet-store mice, has shown upregulation of genes associated with interferon signaling and cytokine production (84). However the levels of secretion of these cytokines might be lower compared to models using systemic injection of microbes. The lack of increased proliferation of HSCs might also be due to a lack of sufficient cytokine response to immediately drive them out of dormancy. Nevertheless, that does not mean that there are absolutely no effects on HSCs. The consequences of any elevation in cytokine response could come into play as the co-housed mice or the wildling mice age further than the observational duration in the experiments conducted in this thesis. The effects of any chronic but milder inflammation and cytokine release might accumulate over time. Hence, longer term monitoring of these mice is required to completely rule out any involvement of bone marrow response in the natural but milder infections observed in the wildling and co-housed mice.

### 6.2.4. Acquiring wild microbiota infections during adulthood negatively affects HSC fitness while acquiring infections neonatally preserves HSC fitness

Chronic infections and inflammation are known to have detrimental effects on HSCs by reducing their functional potency (30,76). This reduction in the fitness of HSCs due to chronic infections has been linked to a push of the dormant HSCs into active proliferation (28,40). Dormant HSCs driven into multiple rounds of proliferation leads to an accumulation of DNA damage and the subsequent depletion of the dormant HSC pool, causing functional defects in engraftment during transplantation (28). Hence, I subject the bone marrow from co-housed (2 months acute and 6 months long-term infections) and the wildling mice to competitive transplantation assay which is a gold standard to assess HSC fitness.

As expected with acute infections, bone marrow from mice that were co-housed only for 2 months did not show any significant long-term engraftment defects. This shows that acute transfer of wild microbiota does not alter HSC function. On the other hand, bone marrow from mice co-housed for a longer duration of 6 months, showed a significant reduction in long-term engraftment potential in the peripheral blood of recipient mice. However, there was no reduced percentage of engraftment of immunophenotypically-defined LT-HSCs in the bone marrow. This means that the

reduced long-term engraftment was a consequence of a reduction in the number of functional HSCs capable of producing all the blood lineages. The observation is in line with previous models of chronic infections, even though there was no alteration in the absolute number of HSCs due to co-housing. However, it has to be noted here that the loss of HSC function was not as dramatic as that shown in previous infection models such as *Mycobacterium avium* (30). This is not surprising considering the overall milder infection response and the lack of a bone marrow response observed with the co-housing model in general. The intriguing finding however was that co-housing mediated infections does not lead to any changes in the proportion of dormant LT-HSCs, as observed in the BrdU pulse-chase experiment. This would suggest that there might also be a proliferation independent mechanism of loss of HSC fitness in response to infections or inflammation. However, assessing HSC dormancy through BrdU has an important limitation that the labelling itself is replication dependent and hence does not label all the dormant HSCs to begin with. Hence, these unlabelled dormant HSCs could still proliferative in response to infections, which cannot be detected here since they were not initially labelled with BrdU. The loss of these deeply dormant HSCs that are not in our detection window, could be contributing to the reduction in HSC fitness observed during 6 months of co-housing.

Bone marrow from the wildling mice were also subject to competitive transplantation assay similar to that for the long-term co-housed mice. The observation of reduced HSC function with long-term co-housing even without significant bone marrow response led to the expectation of a similar observation in the wildling mice. These mice have had the colonized infections for a longer time than the co-housed mice as the wild microbiota are vertically transmitted. This led to the hypothesis that the wildling mice would also exhibit reduced HSC function to the same degree or worse than the co-housed mice. Surprisingly, the bone marrow from wildling mice did not show any defects in long-term engraftment in the competitive transplantation assay. Hence the LT-HSCs in the wildling mice do not show any functional decline in terms of producing the different hematopoietic lineages or in terms of competitive disadvantage in bone marrow engraftment. The colonization of infectious microbiota from birth somehow appears to preserve HSC fitness in the wildling mice rather than reduce it. This differential effect of neonatal versus adult exposure to infections on HSC functional potency is a novel finding and there is a lack of previous studies in literature looking at this phenomenon, to the best of my knowledge. This observation also provokes further questions as to whether there are

differences in age-associated hematopoiesis in the wildling mice compared to clean SPF laboratory mice. Aging in laboratory mice is normally associated with increase in the number of immunophenotypic HSCs and reduced HSC functional potency (232). However, these studies were performed in mice housed under SPF conditions and hence wildling mice could have different dynamics of hematopoietic aging.

## 6.3. Application of the co-housing model to study specific hematopoietic questions

Having validated the co-housing model to study both acute and chronic infections in mice, I asked the question as to whether this model can be used to answer other questions related to infection-associated hematopoiesis. For this I chose a couple of interesting questions – 1) How does infection response differ among different strains of mice used in biological research?, and 2) How does infection-stress mediate hematopoiesis under mutations associated with clonal hematopoiesis?

### 6.3.1. Infection response in laboratory mice can be mouse strain dependent

Mouse strains used for biological research are aplenty and can have biological differences that facilitate their effective use in a particular research topic. The inbred C57BL/6 mouse strain is among the most commonly used mouse strains in research. Multitude of research on hematopoiesis has been performed with this mouse strain, including previous studies on infection-models assessing hematopoietic stem cells (30,40,76,228). DBA2/J is another inbred mouse strain used in research and there have been various studies comparing their HSPCs to those of C57BL/6 mice. The DBA2/J mice have been shown to have an increased number of immunophenotypic HSCs in the bone marrow compared to C57BL/6 mice (233). Studies have also shown that HSCs from DBA2/J mice subjected to 5-FU treatment induced myeloablation, recovered much faster to replenish the peripheral blood cells (234). There are also differences observed in age-associated HSC phenotypes between the two mouse strains (235,236). Such differences in steady-state and stress-hematopoiesis between these mouse strains prompted me to assess how co-housing mediated infection response can vary among them.

To study the strain dependent responses to infections, I co-housed the DBA2/J mice with the wildling mice similar to that with C57BL/6 mice. In contrast to C57BL/6 mice, the DBA/2J mice did not exhibit any increase in the number of peripheral blood immune cells after 2 months from the start of co-housing. This lack of peripheral immune response suggests differential effects of the co-housing mediated infections on different mouse strains. Different mouse strains having differential immune responses to infections is however not a new observation. BALB/c and DBA2/J mice for example, have been shown to act differently in terms of microbial clearance and immune response when infected with *Mycobacterium bovis* (237). Also, the infections that are transmitted from the wildling mice could be different between the two mouse strains. Different mouse strains could potentially be different in terms of their pervasiveness to a particular microbe. Since the wildling mice themselves are of a C57BL/6 background, there might be a more efficient transmission of the whole spectrum of microbiota to the C57BL/6 mice compared to DBA/2J mice during co-housing. Microbiological testing was not performed on the DBA2/J mice and hence this has to be performed when repeating this co-housing experiment. Furthermore, the gut microbiome composition has also been shown to be different among different mouse strains and this difference in composition can lead to differences in immune outcomes (238). Hence the gut microbiome of these mouse strains should also looked at in future co-housing experiments with the DBA/2J mice. Concurrent with the lack of peripheral blood immune changes following co-housing, the DBA/2J mice also did not show any changes in the HSPC composition in the bone marrow following co-housing. In comparison to the studies done with the C57BL/6 mice in this thesis, the DBA/2J mice were only studied with respect to the acute response to co-housing. Co-housing experiments involving long-term analysis similar to that for the C57BL/6 mice should hence be performed to further validate the co-housing model.

### 6.3.2. Co-housing mediated infections do not accelerate clonal hematopoiesis in mice

During aging, the hematopoietic stem cells in individuals acquire somatic mutations over time. When some of these mutations in the HSCs give them a self-renewal or proliferative advantage, there is an expansion of these HSCs in the bone marrow without any apparent hematological disorders. This phenomenon is referred to as clonal hematopoiesis of indeterminate potential (CHIP). CHIP and its associated

mutations are known to also increase the risk of such individuals towards developing hematopoietic neoplasms (215). Mutations that occur in the epigenetic modulators TET2 and DNMT3A are among the top known CHIP associated mutations (101). Furthermore, inflammation and infection induced proliferation of HSCs with these CHIP mutations have been shown to accelerate clonal hematopoiesis in mice (112,113). Additionally, an inflammatory niche has even been shown to be essential to the survival advantage of Tet2-deficient HSCs (239). Hence I aimed to assess how the co-housing mediated colonization of wild microbiota affects hematopoiesis with mice harboring CHIP mutations in HSCs. A transplantation-based model with wildtype competitor cells was used here to mimic clonal hematopoiesis in humans, where only a subset of the HSCs acquire the mutation. It also allowed us to assess whether there were any increase in competitive advantage for the Tet2-knockout and Dnmt3a mutant cells due to co-housing mediated infections. It has to be noted here that the initial engraftment, even before inducing the mutations with Tamoxifen, was lower than the targeted 50%. This indicated a small engraftment defect in these transgenic cells compared to wildtype competitor cells. The initial engraftment was especially lowest for the Dnmt3a transgenic bone marrow. The Dnmt3a mutant cell chimerism in the peripheral blood also dropped over time in the mice, meaning a long term engraftment issue, and hence the data could be used here to convincingly study the aims stated earlier. Hence further discussion here will be only be based on the Tet2 mice.

All co-housed mice in the experiment showed a small increase in the peripheral blood cell count, validating that the co-housing mediated transmission of infections elicited an immune response in the mice. However, it has to be noted that the increase in blood counts was not as high as with the previous co-housing cohorts in this thesis and also the peripheral blood counts were not persistently high as expected over the observational period. The lack of strong immune response here might potentially be due to a differential immune response in transplanted mice compared to non-transplanted mice. The Tet2 mice showed a small increase in the proportion of Tet2 knockout cells in the peripheral blood at 4 months after co-housing, confirming the competitive advantage of Tet2 knockout cells over wildtype cells observed in other studies (216). However, there was no difference observed between Tet2 co-housed and non co-housed Tet2 control mice, suggesting a lack of acceleration or increase in clonal hematopoiesis outcome due to co-housing mediated infections. There was also no expansion of the Tet2 deficient HSPCs in the bone marrow observed. This is not very

surprising here due to two main reasons. Firstly, the acceleration of clonal hematopoiesis is observed as a result of increased HSC proliferation to inflammatory signals in the bone marrow in previous studies (112). The HSCs have not been found to be more proliferative after co-housing even in wildtype mice in the previous experiments as part of this thesis. Hence, the lack of significant bone marrow response to natural but milder transmission of infections could be playing a role in the lack of altered clonal hematopoiesis. Secondly, the time duration required to show differential effects in terms of clonal hematopoiesis outcome in response to the mild infections here might be much greater than the 4 months time-point of observation in my expeirments. Longer tracking of the co-housed Tet2 mutant mice were however not possible due to problems of dermatitis in the transplanted mice. Hence both the clonal hematopoiesis models used here need to be reevaluated for subsequent co-housing experiments.

## 6.4. Limitations associated with the use of the co-housing model using wildling mice

### 6.4.1. Heterogeneity of infection spectrum in wildling mice and co-housed mice

In a previous study involving co-housing using pet-store mice by Beura *et al*., they could identify multiple pathogens present in the pet-store mouse and there was a good horizontal transmission of various infections to the co-housed laboratory mice (84). However, the pet-store mice used in the study had a high heterogeneity in the range of different infections colonized in them. This is as expected since the source of these pet-store mice are varied and the place of breeding are not really controlled to the extent of laboratory mouse housing environments. With the wildling mice having a laboratory mouse genetic background and being bred under controlled environements, it was expected to have a much more non heterogeneous mixture of different infections. However, this was not observed to be the case. The wildling mice also showed heterogeneity in terms of the different infections present in them, albeit to a lower degree compared to the pet-store mice. The heterogeneity of infections observed in the wildling mice also translated into a heterogeneity of colonized infections present in the co-housed mice, even though different wildling mice were rotated between cages during the co-housing period. This could potentially be affecting

some of the variability seen in the data among different mice in the same experimental group.

Furthermore, many of the microbes present in the wildling mice are commensals present in wild mice and are not very pathogenic compared to other models of infection such as *Mycobacterium avium*, mCMV, *Plasmodium berghei*, etc. There are some pathogenic infections such as *Helicobacter pylori* and murine encephalomyelitis viruses in the wildlings that are also transmitted to the co-housed mice. However, in general the co-housing model used in my study have milder infections and this has to be taken into account when comparing this model to existing infection models.

### 6.4.2. Mites and skin flora associated dermatitis in wildling mice and co-housed mice

The wildling mice and concurrently the co-housed mice experience fur loss and dermatitis after around 8-10 months of age. The dermatitis is even more pronounced and accelerated in transplanted recipient mice, potentially owing to the irradiation process required pre-transplantation. Among the plethora of microbes present in the wildling mice, mite infestations are present in all the wildling mice. Furthermore, the skin flora in the wildling mice is vastly different to that in clean SPF mice (89). All of these are of course also transmitted to the co-housed mice. Dermatitis induced inflammation has been shown in literature to affect hematopoiesis and HSPC biology (240). This makes long-term studies of co-housed mice or the wildling mice problematic, especially with the transplanted mice. Switching to non-transplanted mouse models instead of transplantation based models used in this thesis for studying Tet2 and Dnmt3a mutations could partly reduce the problem here. Also, treating the wildling mice with Ivermectin to remove the mites (241), might help to reduce the dermatitis problem if it is solely associated to the presence of fur mites. However, if the skin flora is the reason for dermatitis, the wildling mice might not be a suitable model for long-term studies, especially to study aging associated research questions.

## 6.5. Conclusions and future perspective

The main aim of this project was to assess a co-housing model for studying the effects of a horizontal and vertical transmission of infections on hematopoiesis and hematopoietic stem cell biology. So called wildling mice which carry a range of wild microbiota and infections were co-housed with clean mice that are specific pathogen-free (SPF), to allow the natural horizontal transmission of infections from the wildling mice to the co-housed clean mice. Effective transmission of a range of wild microbiota and infections was observed during the acute period of 2 months from the start of co-housing. The infections were also found to be persistently present at 6 months after co-housing. Thus, the co-housing model can be a suitable model to study the acute and chronic effects of natural horizontal transmission of infections in mice. The wildling mice, having acquired the infections from birth during breeding, served as a model to study the effects of vertical neonatal transmission of wild microbiota and infections.

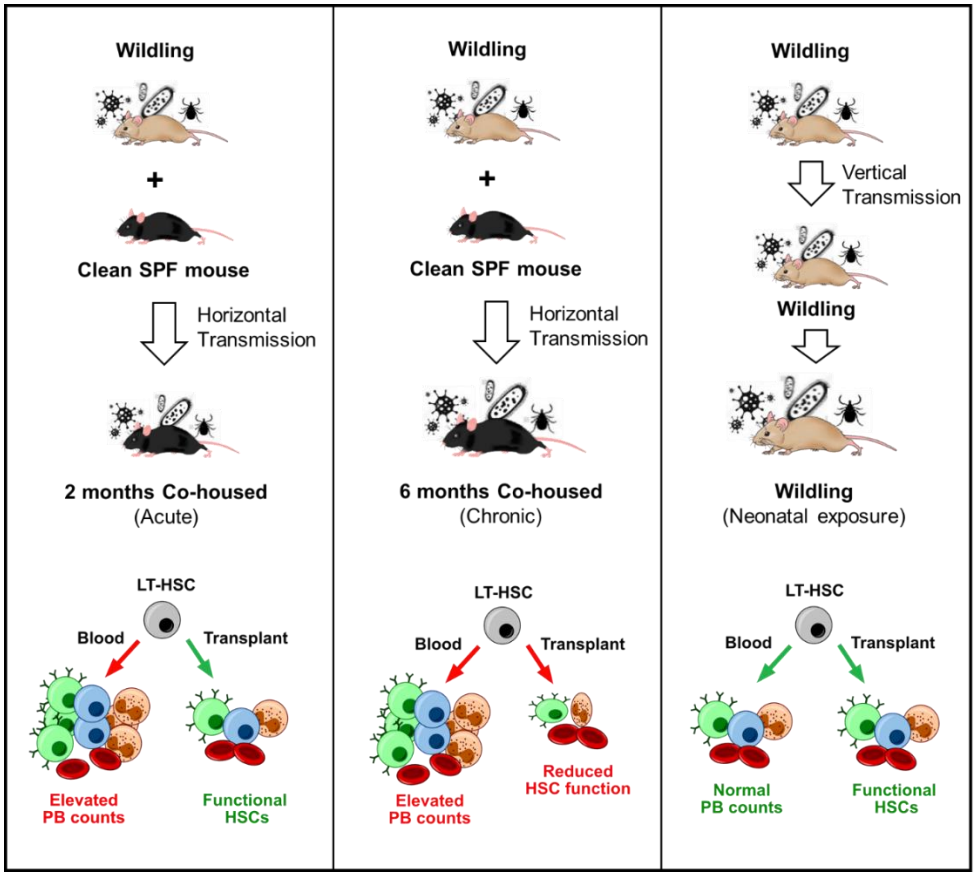


Figure 6.1: Graphical summary of the study performed to assess a co-housing model to understand the effects of horizontal and vertical transmission of infections in mice

Comparing these two modes of transmission of infections, revealed that the co-housed mice have elevated peripheral blood count due to the immune response against the acquired infections, but the wildling mice have normal basal peripheral blood counts even though they have active infections in them. This shows that infecting immunologically naïve mice leads to peripheral immune response, but neonatal exposure to infections normalizes the peripheral immune cell count over time. Furthermore, the chronic presence of infections in the 6 months co-housed mice was found to reduce the functional capacity of the bone marrow LT-HSCs in repopulating the hematopoietic system, while the wildling mice had functionally fit LT-HSCs. Hence, neonatal exposure to infections in mice somehow preserves HSC fitness, compared to acquiring the infections only during adulthood.

Many of the previously studied infection models in mice has shown that chronic infections lead to attrition of HSC function (28,30,76). This has been related to increased proliferation induced in the dormant HSCs in the bone marrow and the subsequent acquisition of replication-stress induced DNA damage. However, the HSCs in the co-housed mice were observed to have functionally compromised HSCs without altered proliferation. This suggests a proliferation-independent mechanism of loss of HSC function in these mice. This is an area that requires further follow up studies such as analysis of the transcriptome of the HSCs in the co-housed mice. Also, the long-term co-housed mice showed no changes in the number of HSPCs or oligopotent progenitors in the bone marrow, even though the number of mature cells in the peripheral blood was persistently high. This would mean that most of the mature cell production could be maintained by more committed precursor cells during a natural progression of infections. This has to be further verified. Single cell RNA-sequencing data has been acquired from the co-housed mice and the data is yet to be analyzed. This would give a better understanding of which bone marrow compartment is driving the required production of the mature blood cells. However, the most intriguing observation in this study was the normal peripheral blood cell counts and the lack of any apparent loss in HSC functional potency in the wildling mice, even though they have had the infections for the longest duration. This observation raises the question, as to whether the presence of infections from birth affects further infection response in the wildling mice. Therefore, follow up experiments where the wildling mice are exposed to further infections is required. The wildling mice could be injected with *Mycobacterium avium* or mCMV for example and the effects can be compared to that

observed in previous studies using those pathogens on clean SPF mice. The single-cell RNA-sequencing data generated from the bone marrow of co-housed and the wildling mice, are also planned to be analyzed to understand the molecular mechanisms behind the preserved HSC fitness in the wildling mice. Overall, the co-housing and wildling mouse models studied in this thesis, has opened the door to study further questions on horizontal versus vertical transfer of infections, a research avenue that has not yet been explored.

# 7 Methods and Materials

## 7.1. Animals

The transgenic mouse lines (Jak2-V617F$^{flox/wt}$, RosaCreERT$^{+/-}$, Dnmt3a-R878H$^{flox/wt}$, Tet2$^{-/-}$, Rosa-EYFP$^{flox/wt}$ and SclCreERT$^{+/-}$), CD45.1 C57BL6/J mice and CD45.1/2 C57BL/6J mice were bred and maintained at the DKFZ animal facility. Jak2-V617F$^{flox/wt}$ mice were crossed with the RosaCreERT$^{+/-}$ mice to generate the Jak2VF mice. Dnmt3a-R878H$^{flox/wt}$ mice were crossed with the SclCreERT$^{+/-}$ and Rosa-EYFP$^{flox/wt}$ mice and are referred to as D3a mice. Tet2$^{-/-}$ mice were crossed with the SclCreERT$^{+/-}$ mice to generate the Tet2 mice. Jak2-V617F, RosaCreERT, Dnmt3a-R878H, Rosa-EYFP and SclCreERT transgenes were heterozygous while the Tet2 transgene was homozygous, in the final experimental mice. All transgenic mice were bred and maintained on a CD45.1 C57BL/6J background. CD45.2 C57BL/6J mice used in experiments were purchased from Janvier labs. CD45.2 C57BL/6NTac mice were purchased from Taconic Biosciences. Wildling mice used in all experiments were kindly provided by the group of Dr. Stephan Rosshart at the Freiburg University hospital.

All mouse experiments performed as part of this thesis was approved by the Regierungspräsidium Karlsuhe under license numbers G-247/18 and G-256/19. Donor mice used for organ sampling purposes were under the approved DKFZ-357 license. All specific pathogen free (SPF) mice were housed in individually ventilated cages (IVC) at the DKFZ animal facility. Wildling mice, co-housed mice and mice transplanted with wildling or co-housed mouse bone marrow, were housed in a flexible film isolator unit at the quarantine room of DKFZ animal facility. All mice were given drinking water and fed *ad libitum*.

## 7.2. Co-housing

Two to three laboratory mice were co-housed with a wildling mouse in the same cage for a period of 3 weeks, and then the wildling mouse was separated from the cage. The wildling mouse in the co-housing cage, was changed to a new wildling mouse once per week, to allow the laboratory mice in each cage to encounter at least 3 different wildling mice during the co-housing period. The co-housing procedure was performed inside a flexible film isolator unit and the co-housed mice were kept there until they were euthanized at the endpoint.

## 7.3. Treatments

### 7.3.1. Tamoxifen

50mg of Tamoxifen powder (Sigma; T-5648-5G) was added to 500µL of 100% ethanol and 4.5mL of sunflower oil (Sigma; S-5007), and dissolved by rotating on a roller mixer for 2 hours at room temperature. This generates tamoxifen solution at a working concentration of 10 mg/mL. Mice were injected with 200µL (2mg) of dissolved tamoxifen solution, intraperitoneally, once per day for 5 consecutive days to activate the Cre recombinase.

### 7.3.2. 5-Bromo-2'-deoxyuridine (BrdU)

***BrdU pulse-chase assay:*** Mice were injected intraperitoneally once with 180µg BrdU (Sigma; B5002) in PBS using a 29-gauge insulin syringe (BD; 324827), following by administering drinking water containing 800 µg/mL BrdU and 2% sucrose, for a period of 14 days. The BrdU water was replenished with freshly prepared BrdU water after 7 days. Following the end of the 14 days the mice were given regular water. BrdU drinking water was prepared by adding 0.8g of BrdU powder (Sigma; B5002-5G) and 20g of sucrose (Sigma; S0389) to 1 litre of water, and heating the solution to 37ºC in a magnetic stirrer until the powder completely dissolves.

***BrdU incorporation assay:*** 10mg/mL solution of BrdU (Sigma; B5002) was prepared by dissolving the powder in PBS. Mice were weighed and injected intraperitoneally

with 100mg/kg-bodyweight of BrdU using a 29-gauge syringe (BD; 324827). The mice were then euthanized 16 hours post-injection.

### 7.3.3. Fedratinib

12 mg/mL stock of Fedratinib solution was prepared by dissolving the Fedratinib powder (MedChemExpress; HY-10409) in a vehicle solution of 10% DMSO (Sigma; D2650) + 40% PEG400 (Hölzel Diagnostika; S6705) + 5% Tween-80 (Sigma; P1754) + 35% PBS (Sigma; D8537). The solutions in the vehicle were added to the powder in order, with vortexing after each component. Mice were weighed and treated with 120mg/kg-bodyweight of Fedratinib solution for 4 weeks (5 consecutive days per week) through oral gavage using a 22-gauge curved gavage needle. The vehicle control mice were administered with 10µL/g-bodyweight of vehicle solution through oral gavage for the same period of time.

### 7.3.4. Pegylated-IFNα

Pegylated murine IFNα (mP1101) was kindly provided by PharmaEssentia as part of a material transfer agreement (PEC No.199900600). 30µg/mL stock solution of peg-IFNα was diluted to 3µg/mL with PBS containing 0.1% BSA. Mice were injected subcutaneously with 200µL (600ng dosage) or with 100µL (300ng dosage) of diluted peg-IFNα solution using a 29-gauge insulin syringe (BD; 324827) once per week for a period of 4 weeks.

## 7.4. Sample collection

### 7.4.1. Whole murine blood and serum

***Whole blood:*** The facial vein of the mouse was punctured using a 23-gauge needle (CHIRANA T.Injecta; CH23100). 60-100µL of blood was collected into a K3 EDTA-coated tube (Sarstedt; 20.1278). 30µL of blood was used to assess the complete blood count using ScilVet abc-Plus+ veterinary blood cell counter (Scil GmbH). Rest of the blood was used for flow cytometry analysis.

***Serum:*** The facial vein of the mouse was punctured using a 23-gauge needle (CHIRANA T.Injecta; CH23100). 100µL of blood was collected into a serum-tube (Sarstedt; 20.1280). The collected blood was allowed to stand at room temperature for 20 minutes at room temperature or at 4ºC for 2 hours, to allow blood coagulation. The tubes were then centrifuged at 2000xg for 10min at 4ºC. The serum in the supernatant was collected into a fresh 1.5mL Eppendorf tube for further analyzes.

### 7.4.2. Mouse bone marrow cells

Mice were euthanized by cervical dislocation and the thoracic vertebrae, femur, ilium and tibia were dissected. The cells from the femur, ilium and tibia were collected by flushing the bones with a 2mL solution of PBS (Sigma; D8537) supplemented with 2% FBS (Gibco), using a 23-gauge needle (CHIRANA T.Injecta; CH23100) and 1mL syringe (Henke Sass Wolf; 5010-200V0). The cell suspension was passed through a 5mL strainer-cap round-bottom tube (Falcon; 352235) and 30µL of the suspension was analyzed using the ScilVet abc-Plus+ cell counter to assess the number of cells obtained.

To obtain larger quantities of cells from the isolated bones, the vertebrae and the leg bones were homogenized three times in 5mL IMDM medium (Thermo Fisher; 21980065) using a mortar and pestle. The cell suspension obtained was filtered through a 40µm cell-strainer (Greiner Bio-One; 542140) and centrifuged at 1500rpm for 5min. The supernatant was discarded and the cell pellet was resuspended in 5mL of PBS/FBS solution. 30µL of the cell suspension was counted using the ScilVet abc-Plus+ to assess the number of cells obtained.

### 7.4.3. Mouse spleen cells

Mice were euthanized by cervical dislocation and the spleen was collected. The spleen was immediately weighed using a weighing scale. The isolated spleen was then crushed on top of a 40µm cell-strainer (Greiner Bio-One; 542140) in 5mL IMDM (Thermo Fisher; 21980065) using the plunger of a 2.5mL syringe. The filtered solution was centrifuged at 1500rpm for 5min. The supernatant was discarded and the cell pellet was resuspended in 3mL of PBS/FBS solution. 30µL of the cell suspension was counted using the ScilVet abc-Plus+ counter to assess the number of cells obtained.

### 7.4.4. Samples for microbiological analysis of mice

Peripheral blood serum, oral swab, fur swab, fecal pellet, anal sample and diverse organs (lung, brain, caecum, jejunum, colon and lymph nodes) were collected from the co-housed and wildling mice. The samples were submitted to the Microbiology department of the DKFZ animal facility for analysis. The serum samples were used to assess the presence of antibodies against common mouse pathogens. The organs, oral swab, fur swab and fecal pellet samples were subjected to microbial culture mediums and PCR tests to assess the presence of live mouse pathogens. All analyzes of these samples were performed by the group of Dr. Katja Schmidt using standardized protocol as part of the DKFZ animal facility.

## 7.5. Bone marrow transplantation

### 7.5.1. Bone marrow preparation

For generating chimeric Jak2VF (also referred to as Jak2-mutant in thesis) and RosaCre mice, bone marrow from Jak2V617F$^{flox/wt}$ x RosaCre$^{+/-}$ or RosaCre$^{+/-}$ mice were mixed with bone marrow from wildtype competitor mice in a 1:2 ratio (VF/Cre:WT). The mixed cell suspension was adjusted to a concentration of 6x10$^6$ cells/200µL in PBS.

For generating chimeric Tet2, D3a and SclCre mice, bone marrow from Tet2$^{-/-}$ x SclCreERT$^{+/-}$, Dnmt3a-R878H$^{flox/wt}$ x SclCreERT$^{+/-}$ x Rosa-EYFP$^{flox/wt}$ or SclCreERT$^{+/-}$ mice were mixed with bone marrow from wildtype competitor mice in a 1:1 ratio. The mixed cell suspension was adjusted to a concentration of 8x10$^6$ cells/200µL in PBS.

For competitive bone marrow transplantation assay, bone marrow from experimental mice were mixed with bone marrow from wildtype competitor mouse in a 1:1 ratio. The mixed cell suspension was adjusted to a concentration of 6x10$^6$ cells/200µL in PBS.

### 7.5.2. Irradiation and transplantation

Recipient mice were conditioned through total body irradiation of two doses of 500 Rad each, using a cesium irradiation source. There was a four hour time interval between the two doses. Following irradiation the mice were administered with 90mg/kg-bodyweight/day of Sulfamethoxazole/Trimethoprim through drinking water, for a

period of three weeks. This was to prevent any irradiation-associated infections. The conditioned recipient mice were subject to bone marrow transplantation within 24 hours from the second dose of irradiation. For this, the mice were briefly exposed to infrared light to dilate the blood vessels in the tail, and were placed inside a restrainer. 200µL of prepared bone marrow cell mixture in PBS, was injected into the tail vein using a 29-gauge insulin syringe (BD; 324827).

## 7.6. Flow cytometry analysis of peripheral blood

30µL of collected whole blood was stained with 70µL of prepared antibody staining mix. The antibody mix was prepared by adding the required volume of respective antibodies in a solution of PBS/FBS. Antibodies used for staining blood for different experiments were as given in **Tables 7.1 to 7.3**. The final concentration of the antibodies were determined by titration analysis for each vial of antibody used. The blood-antibody mix was incubated at 4ºC for 20min. 1mL of Ammonium-Chloride-Potassium (ACK)-lysis buffer (Lonza; 10-548E) was added and further incubated at room temperature for 10min with regular vortexing. The mixture was centrifuged at 3000rpm for 5min in a table-top centrifuge and the supernatant was discarded. The cell-pellet was washed once with 1mL of PBS/FBS and was resuspended in 100µL of PBS/FBS containing either DAPI (1:1000 dilution of 1mg/mL stock) or 7-AAD (1:200 dilution of 1mg/mL stock), as stipulated in **Tables 7.1 to 7.3**. The samples were then filtered through a 40µm filter and subjected to flow cytometric analysis using a LSR-II or a LSR-Fortessa (BD Biosciences) flow cytometer.

## 7.7. Flow cytometry analysis of bone marrow and spleen

Required number of bone marrow and spleen cells (5-8 million for progenitor staining and 10-15 million for HSPC staining) were aliquot and centrifuged at 3000rpm for 5min in a table-top centrifuge. The cell pellet was resuspended in antibody staining mix. The antibody mix was prepared by adding the required volume of respective antibodies in a solution of PBS/FBS. Antibodies used for staining cells for different experiments were as given in **Tables 7.4 to 7.9**. The final concentration of the antibodies were determined by titration analysis for each vial of antibody used. The cells-antibody mix was incubated at 4ºC for 20min. 1mL of ACK-lysis buffer (Lonza;

10-548E) was added and further incubated at room temperature for 3min. The mixture was then centrifuged at 3000rpm for 5min in a table-top centrifuge and the supernatant was discarded. The cell-pellet was washed once with 1mL of PBS/FBS and was resuspended in 300µL of PBS/FBS. The samples were then filtered through a 40µm filter and subjected to flow cytometric analysis using a LSR-II or a LSR-Fortessa (BD Biosciences) flow cytometer.

**Table 7.1: Antibody panel to assess peripheral blood chimerism in transplanted mice**

| Antigen | Fluorophore | Clone | Company/Ref.No. |
|---|---|---|---|
| Gr-1 | APC-Cy7 | RB6-BC5 | eBioscience (47-5931-82) |
| CD11b | APC-Cy7 | M1/70 | eBioscience (47-0112-82) |
| B220 | APC-Cy7 | RA3-6B2 | eBioscience (47-0452-82) |
| B220 | PE-Cy7 | RA3-6B2 | eBioscience (25-0452-82) |
| CD4 | PE-Cy7 | GK1.5 | eBioscience (25-0041-82) |
| CD8 | PE-Cy7 | 53-6.7 | eBioscience (25-0081-82) |
| CD45.1 | Pacific Blue | A20 | Biolegend (110722) |
| CD45.2 | APC | 104 | eBioscience (17-0454-82) |
| Dead cells | 7-AAD | - | Life Technologies (A1310) |

**Table 7.2: Antibody panel for mature cells and T-cell subsets in peripheral blood**

| Antigen | Fluorophore | Clone | Company/Ref.No. |
|---|---|---|---|
| CD4 | PE | GK1.5 | eBioscience (12-0041-83) |
| CD8 | BUV395 | 53-6.7 | BD Biosciences (563786) |
| B220 | APC | RA3-6B2 | eBioscience (17-0452-83) |
| CD11b | PE-Cy5 | M1/70 | eBioscience (15-0112-83) |
| Ly6C | APC-Cy7 | HK1.4 | Biolegend (128026) |
| CD62L | PE-Cy7 | MEL-14 | Biolegend (104418) |
| CD44 | FITC | IM7 | Biolegend (103006) |
| Dead cells | DAPI | - | Thermo Fisher (62248) |

**Table 7.3: Antibody panel for mature cells in competitive transplantation assay**

| Antigen | Fluorophore | Clone | Company/Ref.No. |
|---|---|---|---|
| CD4 | FITC | RM4-5 | eBioscience (11-0042-82) |
| CD8 | PE-Cy7 | 53-6.7 | eBioscience (25-0081-82) |
| B220 | APC-Cy7 | RA3-6B2 | eBioscience (47-0452-82) |
| CD11b | APC | M1/70 | eBioscience (17-0112-83) |
| Gr-1 | PE | RB6-8C5 | eBioscience (12-5931-81) |
| CD45.1 | Pacific Blue | A20 | Biolegend (110722) |
| CD45.2 | Alexa-700 | 104 | eBioscience (56-0454-82) |
| Dead cells | 7-AAD | - | Life Technologies (A1310) |

**Table 7.4: Antibody panel for HSPCs in transplanted Jak2-mutant mice**

| Antigen | | Fluorophore | Clone | Company/Ref.No. |
|---|---|---|---|---|
| Lineage | CD4 | APC | GK1.5 | eBioscience (17-0041-83) |
| | CD8 | APC | 53-6.7 | eBioscience (17-0081-83) |
| | B220 | APC | RA3-6B2 | eBioscience (17-0452-83) |
| | CD11b | APC | M1/70 | eBioscience (17-0112-83) |
| | Gr-1 | APC | RB6-BC5 | eBioscience (17-5931-82) |
| | Ter119 | APC | TER-119 | eBioscience (17-5921-81) |
| cKit | | BV711 | 2B8 | Biolegend (105835) |
| Sca1 | | APC-Cy7 | D7 | BD Biosciences (560654) |
| CD150 | | PE-Cy5 | TC15-12F12.2 | Biolegend (115912) |
| CD48 | | PE-Cy7 | HM48-1 | Biolegend (103424) |
| CD34 | | Alexa-700 | RAM34 | eBioscience (CUST03152) |
| CD45.1 | | PE | A20 | Biolegend (110708) |
| CD45.2 | | FITC | 104 | eBioscience (11-0454-85) |

**Table 7.5: Antibody panel for HSPCs in transplanted mice Tet2 and D3a mice**

| Antigen | | Fluorophore | Clone | Company/Ref.No. |
|---|---|---|---|---|
| Lineage | CD4 | eFluor450 | GK1.5 | eBioscience (48-0041-82) |
| | CD8 | eFluor450 | 53-6.7 | eBioscience (48-0081-82) |
| | B220 | eFluor450 | RA3-6B2 | eBioscience (48-0452-82) |
| | CD11b | eFluor450 | M1/70 | eBioscience (48-0112-82) |
| | Gr-1 | eFluor450 | RB6-BC5 | eBioscience (48-5931-82) |
| | Ter119 | eFluor450 | TER-119 | eBioscience (48-5921-82) |
| cKit | | BV711 | 2B8 | Biolegend (105835) |
| Sca1 | | APC-Cy7 | D7 | BD Biosciences (560654) |
| CD150 | | PE-Cy5 | TC15-12F12.2 | Biolegend (115912) |
| CD48 | | PE-Cy7 | HM48-1 | Biolegend (103424) |
| CD34 | | Alexa-700 | RAM34 | eBioscience (CUST03152) |
| CD45.1 | | PE | A20 | Biolegend (110708) |
| CD45.2 | | APC | 104 | eBioscience (17-0454-82) |

**Table 7.6: Antibody panel for HSPCs in non-transplanted mice**

| Antigen | | Fluorophore | Clone | Company/Ref.No. |
|---|---|---|---|---|
| Lineage | CD4 | eFluor450 | GK1.5 | eBioscience (48-0041-82) |
| | CD8 | eFluor450 | 53-6.7 | eBioscience (48-0081-82) |
| | B220 | eFluor450 | RA3-6B2 | eBioscience (48-0452-82) |
| | CD11b | eFluor450 | M1/70 | eBioscience (48-0112-82) |
| | Gr-1 | eFluor450 | RB6-BC5 | eBioscience (48-5931-82) |
| | Ter119 | eFluor450 | TER-119 | eBioscience (48-5921-82) |
| cKit | | BV711 | 2B8 | Biolegend (105835) |
| Sca1 | | APC-Cy7 | D7 | BD Biosciences (560654) |
| CD150 | | PE-Cy5 | TC15-12F12.2 | Biolegend (115912) |
| CD48 | | PE-Cy7 | HM48-1 | Biolegend (103424) |
| CD34 | | FITC | RAM34 | eBioscience (11-0341-82) |

**Table 7.7: Antibody panel for progenitor cells in transplanted D3a and Tet2 mice**

| Antigen | | Fluorophore | Clone | Company/Ref.No. |
|---|---|---|---|---|
| Lineage | CD4 | eFluor450 | GK1.5 | eBioscience (48-0041-82) |
| | CD8 | eFluor450 | 53-6.7 | eBioscience (48-0081-82) |
| | B220 | eFluor450 | RA3-6B2 | eBioscience (48-0452-82) |
| | CD11b | eFluor450 | M1/70 | eBioscience (48-0112-82) |
| | Gr-1 | eFluor450 | RB6-BC5 | eBioscience (48-5931-82) |
| | Ter119 | eFluor450 | TER-119 | eBioscience (48-5921-82) |
| cKit | | BV711 | 2B8 | Biolegend (105835) |
| Sca1 | | APC-Cy7 | D7 | BD Biosciences (560654) |
| CD150 | | PE-Cy5 | TC15-12F12.2 | Biolegend (115912) |
| CD48 | | PE-Cy7 | HM48-1 | Biolegend (103424) |
| CD34 | | Alexa-700 | RAM34 | eBioscience (CUST03152) |
| CD45.1 | | PE | A20 | Biolegend (110708) |
| CD45.2 | | PE-Cy7 | 104 | Biolegend (109830) |

**Table 7.8: Antibody panel for progenitor cells in non-transplanted mice**

| Antigen | | Fluorophore | Clone | Company/Ref.No. |
|---|---|---|---|---|
| Lineage | CD4 | eFluor450 | GK1.5 | eBioscience (48-0041-82) |
| | CD8 | eFluor450 | 53-6.7 | eBioscience (48-0081-82) |
| | B220 | eFluor450 | RA3-6B2 | eBioscience (48-0452-82) |
| | CD11b | eFluor450 | M1/70 | eBioscience (48-0112-82) |
| | Gr-1 | eFluor450 | RB6-BC5 | eBioscience (48-5931-82) |
| | Ter119 | eFluor450 | TER-119 | eBioscience (48-5921-82) |
| cKit | | BV711 | 2B8 | Biolegend (105835) |
| Sca1 | | APC-Cy7 | D7 | BD Biosciences (560654) |
| CD16/32 | | APC | 93 | eBioscience (17-0161-82) |
| CD34 | | FITC | RAM34 | eBioscience (11-0341-82) |
| CD55 | | PE | RIKO-3 | Biolegend (131803) |
| CD127 | | PE-Cy7 | SB/199 | BD Biosciences (560733) |

**Table 7.9: Antibody panel for erythroid cells in chimeric Jak2-mutant mice**

| Antigen | | Fluorophore | Clone | Company/Ref.No. |
|---|---|---|---|---|
| Lineage | CD4 | APC-Cy7 | GK1.5 | Biolegend (100414) |
| | CD8 | APC-Cy7 | 53-6.7 | Biolegend (100714) |
| | B220 | APC-Cy7 | RA3-6B2 | eBioscience (47-0452-82) |
| | CD11b | APC-Cy7 | M1/70 | eBioscience (47-0112-82) |
| | Gr-1 | APC-Cy7 | RB6-BC5 | eBioscience (47-5931-82) |
| Ter119 | | eFluor450 | TER-119 | eBioscience (48-5921-82) |
| CD71 | | PE | R17 217.1.4 | eBioscience (12-0711-83) |

*Note: No ACK-lysis was performed for this staining*

## 7.8. Enrichment of lineage negative bone marrow cells

Total bone marrow cells were obtained by crushing the leg bones and the spine, and finally suspended in 10mL of PBS/FBS. 100µL of lineage antibody cocktail **(Table 7.10)** was added to the cell suspension and incubated at 4ºC in a roller for 30min. The cells were washed with PBS/FBS by centrifugation. The cell pellet was then resuspended in PBS/FBS, and pre-washed anti-rat IgG-coated magnetic Dynabeads from the Dynabeads Untouched Mouse T Cells kit (Life Technologies; 11415D) was added to the cell suspension (1mL beads per mouse). The cell-Dynabead mix was incubated at 4ºC in a roller for 30min. The mix was then placed on a magnetic rack and the supernatant was collected. The collected supernatant containing the lineage depleted fraction was washed with PBS/FBS and resuspended in 1mL PBS/FBS. 30µL of the cell suspension was used for assessing the number of cells obtained using the ScilVet abc-Plus+ counter. The lineage-depleted bone marrow cells were generated only for chimeric Jak2-mutant mice and subsequently used for BrdU staining.

**Table 7.10: Composition of lineage antibody cocktail for lineage cell depletion**

| Antigen | Isotype | Clone | Company |
|---|---|---|---|
| CD3 | Hamster IgG1 /K | 145.2C11 | Self-made |
| CD4 | Rat IgG2b /K | YTS 191.1 | Self-made |
| CD8 | Rat IgG2b /K | YTS 169.4 | Self-made |
| CD11b | Rat IgG2b /K | M1/70 | Self-made |
| Gr-1 | Rat IgG2a /K | RB68C5 | Self-made |
| B220 | Rat IgG2a /K | RA3632 | Self-made |
| Ter119 | Rat IgG2b /K | TER119 | Self-made |

## 7.9. Flow cytometry of cells from BrdU treated mice

BrdU staining was performed using components from BrdU staining-kit (BD Biosciences; 552598), with a modified version of the kit protocol. 15 x $10^6$ bone marrow or spleen cells were suspended in 300µL of antibody mix for surface staining. The antibody mix was prepared by adding the required volume of respective antibodies in a solution of PBS/FBS. Antibodies used for staining cells for different experiments were as given in **Tables 7.11 and 7.12**. The final concentration of the antibodies were determined by titration analysis for each vial of antibody used. The cells-antibody mix was incubated at 4ºC for 20min. 1mL of ACK-lysis buffer (Lonza; 10-548E) was added and further incubated at room temperature for 3min. The mixture was then centrifuged at 3000rpm for 5min in a table-top centrifuge and the supernatant was discarded. The cell-pellet was washed once with 1mL of PBS/FBS and was resuspended in 200µL of Cytoperm/Cytofix buffer. The mix was incubated on ice for 25min and then washed once with 1mL of Perm/wash buffer. The pellet was then resuspended in 200uL Cytoperm-Permeabilization-Plus buffer and incubated for 10min on ice. The cells were again washed with 1mL of perm/wash buffer and the pellet was suspended in 200µL Cytofix/Cytoperm buffer. The mixture was incubated on ice for 5 min and the cells were washed with 1mL Perm/wash buffer. Cell pellet was then resuspended in 200µL of diluted DNase solution (65µL of 1mg/mL DNase + 135µL PBS), and incubated at 37ºC for one hour. The cells were then washed with 1mL of Perm/wash buffer and resuspended in anti-BrdU antibody solution (1:200 dilution). The cell-antibody mix was incubated overnight at 4ºC. The cells were then washed

once with PBS/FBS, resuspended in 300µL PBS/FBS and analyzed in a LSR-Fortessa flow cytometer (BD Biosciences).

**Table 7.11: Antibody mix for HSPC-staining of BrdU treated Jak2-mutant mice**

| Antigen | | Fluorophore | Clone | Company/Ref.No. |
|---|---|---|---|---|
| Lineage | CD4 | FITC | RM4-5 | eBioscience (11-0042-82) |
| | CD8 | FITC | 53-6.7 | eBioscience (11-0081-82) |
| | B220 | FITC | RA3-6B2 | eBioscience (11-0452-82) |
| | CD11b | FITC | M1/70 | eBioscience (11-0112-85) |
| | Gr-1 | FITC | RB6-8C5 | eBioscience (11-5931-85) |
| | Ter119 | FITC | TER-119 | eBioscience (11-5921-82) |
| cKit | | BV711 | 2B8 | Biolegend (105835) |
| Sca1 | | APC-Cy7 | D7 | BD Biosciences (560654) |
| CD150 | | PE-Cy5 | TC15-12F12.2 | Biolegend (115912) |
| CD48 | | PE-Cy7 | HM48-1 | Biolegend (103424) |
| CD34 | | Alexa-700 | RAM34 | eBioscience (CUST03152) |
| CD45.1 | | PE | A20 | Biolegend (110708) |
| CD45.2 | | Pacific Blue | 104 | Biolegend (109820) |

**Table 7.12: Antibody mix for HSPCs from BrdU-labelled non-transplanted mice**

| Antigen | | Fluorophore | Clone | Company/Ref.No. |
|---|---|---|---|---|
| Lineage | CD4 | eFluor450 | GK1.5 | eBioscience (48-0041-82) |
| | CD8 | eFluor450 | 53-6.7 | eBioscience (48-0081-82) |
| | B220 | eFluor450 | RA3-6B2 | eBioscience (48-0452-82) |
| | CD11b | eFluor450 | M1/70 | eBioscience (48-0112-82) |
| | Gr-1 | eFluor450 | RB6-BC5 | eBioscience (48-5931-82) |
| | Ter119 | eFluor450 | TER-119 | eBioscience (48-5921-82) |
| cKit | | BV711 | 2B8 | Biolegend (105835) |
| Sca1 | | APC-Cy7 | D7 | BD Biosciences (560654) |
| CD150 | | PE-Cy5 | TC15-12F12.2 | Biolegend (115912) |
| CD48 | | PE-Cy7 | HM48-1 | Biolegend (103424) |
| CD34 | | FITC | RAM34 | eBioscience (11-0341-82) |
| CD16/32 | | PE | 93 | eBioscience (12-0161-82) |

## 7.10. Analysis of flow cytometry data

The analysis of all data from the flow cytometers were performed using FlowJo Version 10 (FlowJo LLC). The representative gating schemes for different analyses are provided as figures in the two results sections **(Figures 3.2C, 3.3B, 5.3, 5.6, 5.8 and 5.32A)**.

## 7.11. Statistical analysis

Statistical analysis for all experimental data were performed using paired or unpaired parametric t-tests in Graphpad Prism Version 9.1 (Graphpad Software Inc.). The sample size for each experimental group is as mentioned in the respective figure legends. The symbols * represents $P<0.05$, ** represents $P<0.01$, *** represents $P<0.001$ and **** represents $P<0.0001$. Lack of symbol or 'ns' represents no significant differences. All data values in the figures are presented as mean-values with error bars representing standard-deviation (SD).

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# Abbreviations

| | Abbreviation | Definition |
|---|---|---|
| | ˚C | Degree Celsius |
| | µg | Microgram |
| | µL | Micro-litres |
| | µm | Micro-meter |
| | 5-FU | 5-Fluorouracil |
| | 7-AAD | 7-Aminoactinomycin D |
| A | ACK | Ammonium-Chloride-Potassium |
| | AML | Acute Myeloid Leukemia |
| | APC | Allophycocyanin |
| | APC-Cy7 | Allophycocyanin cyanine 7 |
| | ASXL2 | Additional Sex Combs-Like Protein 2 |
| B | BM | Bone Marrow |
| | BrdU | 5-Bromo-3'-deoxyuridine |
| | BSA | Bovine serum albumin |
| | BW | Body-weight |
| C | CALR | Calreticulin |
| | CHIP | Clonal Hematopoiesis of Indeterminate Potential |
| | CLP | Common Lymphoid Progenitor |
| | CML | Chronic Myeloid Leukemia |
| | CMP | Common Myeloid Progenitor |
| | CSC | Cancer stem cell |
| | Ctrl | Control |
| D | DAPI | 4′,6-diamidino-2-phenylindole |
| | DKFZ | Deutsches Krebsforschungszentrum |
| | dL | Deci-litre |
| | DMSO | Dimethyl Sulfoxide |
| | DNA | Deoxyribonucleic acid |
| | DNase | Deoxyribonuclease |
| | DNMT3A | DNA methyltransferase 3 alpha |
| | dsRNA | Double-stranded Ribonucleic acid |
| E | EDTA | Ethyline diamine tetra acetic acid |
| | EMA | European Medicines Agency |

| | | |
|---|---|---|
| | EMH | Extramedullary hematopoiesis |
| | EPO | Erythropoietin |
| | Ery | Erythrocyte |
| | ET | Essential thrombocythemia |
| | EYFP | Enhanced Yellow Fluorescent Protein |
| F | FBS | Fetal Bovine Serum |
| | FDA | Food and Drug Administration |
| | FITC | Fluorescein isothiocyanate |
| G | g | Gram |
| | G-CSF | Granulocyte – Colony Stimulating Factor |
| | GFP | Green fluorescent protein |
| | GM-CSF | Granulocyte Macrophage – Colony Stimulating Factor |
| | GMP | Granulocyte-Monocyte Progenitors |
| H | H2B | Histone 2B |
| | Hb | Hemoglobin |
| | HCT | Hematocrit |
| | HSC | Hematopoietic Stem Cells |
| | HSPC | Hematopoietic Stem and Progenitor Cells |
| I | IFN | Interferon |
| | IFNα | Interferon-alpha |
| | IL | Interleukin |
| | IMDM | Iscove's Modified Dulbecco's Medium |
| J | JAK2 | Janus-kinase 2 |
| | JAKi | Janus-kinase inhibitor |
| K | kg | Kilogram |
| L | LCMV | Lymphocyte choriomeningitis virus |
| | Lin | Lineage |
| | LK | Lineage$^-$ cKit$^+$ Sca1$^-$ |
| | LPS | Lipopolysaccharide |
| | LRP | Lineage Restricted Progenitors |
| | LSK | Lineage$^-$ Sca1$^-$ cKit$^+$ |
| | LT-HSC | Long-term hematopoietic stem cells |
| M | mCMV | Murine Cytomegalovirus |
| | MDS | Myelodysplastic Syndrome |
| | MEP | Megakaryocyte-Erythroid Progenitors |
| | mg | Milligram |
| | Min | Minutes |

| | | |
|---|---|---|
| | mL | Millilitre |
| | MPL | Myeloproliferative Leukemia Protein |
| | MPN | Myeloproliferative neoplasm |
| | MPP | Multipotent Progenitors |
| | ng | Nano-gram |
| N | NLRP3 | NACHT, LRR And PYD Containing Protein 3 |
| | NPM1 | Nucleophosmin 1 |
| | PAMP | Pathogen associated molecular patterns |
| | PB | Peripheral blood |
| | PBMC | Peripheral blood mononuclear cells |
| | PBS | Phosphate-buffered saline |
| | PCR | Polymerase Chain Reaction |
| | PE | Phycoerythrin |
| | PE-Cy5 | Phycoerythrin cyanine 5 |
| P | PE-Cy7 | Phycoerythrin cyanine 7 |
| | PEG | Polyethyleneglycol |
| | peg-IFNα | Pegylated-Interferon-alpha |
| | pI:pC | Polyinosinic:polycytidylic acid |
| | PLT | Platelet |
| | PMF | Primary myelofibrosis |
| | PML | Promyelocytic leukemia |
| | PV | Polycythemia vera |
| | R878H | Arginine to Histidine substitution at position 878 |
| | R882H | Arginine to Histidine substitution at position 882 |
| R | RCC | Red cell count |
| | RNA | Ribonucleic acid |
| | rpm | Rotations per minute |
| | RT | Room temperature |
| | SD | Standard Deviation |
| | sec | Seconds |
| S | SPF | Specific pathogen free |
| | STAT | Signal Transducer And Activator Of Transcription |
| | ST-HSC | Short-term hematopoietic stem cells |
| | TET2 | Ten-eleven translocation 2 |
| T | TLR | Toll-like receptor |
| | TNF-α | Tumour Necrosis Factor – alpha |
| | TPO | Thrombopoietin |

| | | |
|---|---|---|
| V | V617F | Valine to Phenylalanine substitution at position 617 |
| | VAF | Variant allele frequency |
| W | WBC | White blood cell |
| | WCC | White cell count |
| | WT | Wild-type |

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# Presentation of work

**Timing of Exposure to Infectious Microbiota Determines HSC Fitness**
Jeyan Jayarajan, Melanie Ball, Dhyani Shah, Esther Rodríguez-Correa, Susanne Lux, Florian Grünschläger, Foteini Fotopoulou, Ana-Matea Mikecin, Simon Haas, Katja Schmidt, Stephan Rosshart, Michael Milsom
*European Hematology Association Congress 2022, Vienna*
*June 2022 (Poster Presentation)*

**Interferon-Alpha Treatment Results in the Depletion of Dormant JAK2-Mutant HSCs in a Murine Model of Polycythemia Vera**
Jeyan Jayarajan, Melanie Ball, Julia Knoch, Ian Ghezzi, Michael Milsom
*European School of Hematology 9th Annual Translational Conference on Myeloproliferative Neoplasms (Virtual)*
*May 2022 (Poster Presentation with Flash-talk)*

**A Co-Housing Model System to Study the Effect of Infection-Stress on Hematopoietic Stem and Progenitor Cells**
Jeyan Jayarajan, Melanie Ball, Dhyani Shah, Esther Rodríguez-Correa, Susanne Lux, Foteini Fotopoulou, Ana-Matea Mikecin, Katja Schmidt, Stephan Rosshart, Michael Milsom
*DKFZ PhD Poster Presentation 2021, Heidelberg*
*November 2021 (Poster presentation)*

**Interferon-α Treatment Results in the Depletion of Dormant JAK2-Mutant HSCs in a Murine Model of Polycythemia Vera**
Jeyan Jayarajan, Melanie Ball, Julia Knoch, Michael Milsom
*European Hematology Association Congress 2021 (Virtual)*
*June 2021 (Oral presentation)*

**A co-housing model system to study the effect of infection-stress on hematopoietic stem cells**

Jeyan Jayarajan, Melanie Ball, Julia Knoch, Michael Milsom

*AMPro Summer School on Aging, Garmisch-Partenkirchen*

*September 2019 (Oral presentation)*

# Contributions to work

The work in this thesis was performed under the supervision of **Dr. Michael Milsom** in the Division of Experimental Hematology at the German Cancer Research Centre (DKFZ) and the Heidelberg Institute for Stem Cells and Experimental Medicine (HI-STEM).

**Melanie Ball** (Experimental Hematology, DKFZ) and **Julia Knoch** (Experimental Hematology, DKFZ) provided the technical support for all the studies in this dissertation, such as mouse breedings, sample collections, mouse treatments, flow cytometry staining, etc.

Flow cytometry and the subsequent data analysis for the experiments on Dnmt3a and Tet2 mutant mice were performed by **Dhyani Shah** (Master student, Experimental Hematology, DKFZ), under my direct supervision.

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