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Identification of novel mechanisms controlling emergency granulopoiesis in hematopoietic stem and progenitor cells

Identifikace nových mechanismů kontrolujících pohotovostní granulopoézu v hematopoetických kmenových a progenitorových buňkách

Diploma thesis

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Prohlášení

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Abstract

Granulocytes represent the first line of defense against bacteria and fungi. Daily production of granulocytes is sustained by steady state granulopoiesis but under stress (e.g., bacterial infection) this program switches to emergency granulopoiesis (EG) which ensures the production of granulocytes at enhanced and accelerated rates. Very little is known about the regulation of EG. In this thesis, we showed that disruption of the β-catenin-TCF/LEF mediated transcription impairs EG *in vivo*. Further, we demonstrated that lipopolysaccharide (LPS) administration in mice induces accumulation of active β-catenin in hematopoietic stem and progenitor cells (HSPCs) as early as 4 hours (H) after stimulation, with highest increase at 24H. This effect was at least partially mediated in a niche independent manner, since LPS stimulation *in vitro* induced β-catenin accumulation in c-Kit+ cells after 2H, with a peak activation at 4H. Using single cell RNA sequencing, we determined the cell cluster dynamics of HSPCs following 4H LPS stimulation. Interestingly, we identified a possible upstream activator of β-catenin in one of the clusters – Wnt10b. Indeed, *Wnt10b* showed a similar expression pattern as EG master regulator *Cebpb* and β-catenin activation, following *in vitro* treatment with LPS. Altogether, our data point at a critical role of the Wnt/β-catenin-TCF/LEF signaling pathway in activation of the EG program at the HSPC level at early stages upon infection.

Key words: Granulocytes, Emergency Granulopoiesis, Inflammation, β-catenin, Hematopoietic Stem and Progenitor Cell

Abstrakt

Granulocyty představují první linii obrany proti bakteriálním a houbovým patogenům. Zatímco bazální granulopoéza zajišťuje každodenní produkci granulocytů, ve stresových podmínkách, jako je bakteriální infekce, je zvýšená a urychlená produkce granulocytů zajišťována pohotovostní granulopoézou (PG). Velmi málo se ví o tom, jak je PG regulovaná. V této práci jsme ukázali, že signalizační dráha Wnt/β-katenin je zásadní pro aktivaci PG *in vivo.* Dále jsme ukázali, že stimulace pomocí lipopolysacharidu (LPS) aktivuje akumulaci aktivního β-kateninu v hematopoetických kmenových a progenitorových buňkách (HKPB) již po 4 hodinách (H), největší nárůst jsme pozorovali 24H po stimulaci. Tuto aktivaci jsme pozorovali také *in vitro*, již 2H po LPS stimulaci c-Kit+ buněk, s největším nárůstem 4H po stimulaci. To dokazuje, že aktivace β-kateninu je alespoň částečně nezávislá na mikroprostředí kostní dřeně. Pomocí single cell RNA sekvenování jsme analyzovali dynamiku jednotlivých sub-populací HKPB 4H po LPS stimulaci. V jedné z populací jsme identifikovali možný aktivátor β-kateninové signalizace – Wnt10b. Nárůst exprese *Wnt10b* jsme detekovali také při *in vitro* LPS stimulacích. Tento nárůst koreloval s aktivací β-kateninu a nárůstem exprese *Cebpb,* hlavního regulátoru pohotovostní granulopoézy. Naše data tak ukazují na zásadní roli signalizace Wnt/β-catenin-TCF/LEF pro program PG na úrovni HKPB v časných stádiích infekce.

Klíčová slova: granulocyty, pohotovostní granulopoéza, zánět, β-katenin, hematopoietická kmenová a progenitorová buňka

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List of abbreviations

5-FU	5-fluorouracyl
BM	Bone marrow
BSA	Bovine serum albumin
CEBP	CCAAT/enhance-binding protein
cGMP	Compact GMP cluster
CMP	Common myeloid progenitor
CSF	Colony stimulating factor
Cy/G	Cyclophosphamide/G-CSF
DC	Dendritic cell
EG	Emergency granulopoiesis
FBS	Fetal bovine serum
FDR	False discovery rate
GMP	Granulocyte monocyte progenitor
GO	Gene ontology
GOBP	GO biological processes
GOCC	GO cellular components
GOMF	GO molecular function
GSEA	Gene set enrichment analysis
H	Hour(s)
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem and progenitor cell
IFN	Interferon
KO	Knock out
LK	Lineage- ckit+
LMPP	Lymphoid-primed multipotent progenitors
LPS	Lipopolysaccharide
LSK	Lineage- ckit+ sca1+
mDC	Myeloid dendritic cells
Meg/E	Megakaryocyte/Erythroid
MEP	Megakaryocyte-erythroid progenitor
MFI	Mean fluorescence intensity
MPP	Multipotent progenitor
MSigDB	Molecular signature database
NES	Normalized enrichment score
NET	Neutrophil extracellular trap
PBS	Phosphate buffered saline
pDC	Plasmacytoid dendritic cell
PID	Pathway interaction database
scRNA-seq	Single cell RNA sequencing
SSG	Steady state granulopoiesis
TF	Transcription factor
TLR	Toll like receptor
UMAP	Uniform manifold approximation and projection
WT	Wild type

1 Introduction

Granulocytes are the most abundant leukocyte population with neutrophils making up to 60% of all white blood cells in adult human. They are part of the innate immune system and represent the first line of defense in our organism. When an infection caused by a bacterium or a fungus occurs, granulocytes are the first cells to be recruited to fight the pathogen. Apart from phagocytosis of the pathogen that leads to the activation of oxidative burst and production of ROS, granulocytes kill the pathogen by degranulation of antimicrobial peptides or by production of neutrophil extracellular traps (NET) [1]. Defects in granulocytic development results in neutropenia, a severe condition, characterized by low numbers of circulating neutrophils that leads to reoccurring infections and possible development of hematological malignancies [2].

Steady state granulopoiesis (SSG) refers to the daily production of granulocytes. The daily rate of granulocytic production in a healthy adult in steady state conditions is 0,5 – 1 x 10^{11} [3]. However, during inflammation there is an increased need for innate immune cells to fight the infection, this is sometimes termed as demand-adapted hematopoiesis and the main hallmark is myeloid skewing. Since mature granulocytes are not able to divide and they undergo cell death at high rates at the site of infection, new cells must be produced from hematopoietic stem and progenitor cells (HSPC) in the bone marrow (BM) at high rates and be flushed out to the blood, through which they travel onto the site of infection. Since this enhanced need for granulocytes cannot be met by steady state granulopoiesis, the program switches to emergency granulopoiesis (EG), which allows for increased and accelerated de novo production of granulocytes in response to an infection in the organism [4].

The changes that happen in EG have been described at the level of mature neutrophils and myeloid progenitors, however very little is known about the changes that occur at the level of hematopoietic stem cells (HSCs) that drive the switch from SSG to EG. Since preliminary results suggested that the β-catenin signaling complex is crucial for both SSG and EG, in the first aim of this thesis we investigated β-catenin signaling complex in the context of EG both *in vitro* and *in vivo*. In the second aim of this thesis, we dissected the changes occurring in HSPCs during EG at single cell level. Altogether, our results identify the early cluster dynamics and transcriptional changes that happen in HSPCs

following the induction of EG using single cell RNA sequencing (scRNA-seq) and demonstrated that the Wnt/ β-catenin signaling pathway is a key mediator of EG.

2 Steady state granulopoiesis

Although neutrophils are the most abundant and are commonly referred to with the general term granulocytes, there are two more, less abundant types of granulocytes: eosinophils, which are important for infections caused by helminths, and the least abundant basophils, which are usually described in connection with allergic reactions [5]. The development of a granulocyte, like any other hematopoietic cell, starts with a hematopoietic stem cell (HSC), and includes steps of lineage commitment (towards the lymphoid or the myeloid lineage) and differentiation (Figure 1A). HSCs (Lin- c-Kit+ Sca1+ CD48- CD150+) have the ability of self-renewal and can repopulate the whole hematopoietic system upon BM cell depletion. HSCs give rise to multipotent progenitors (MPPs, Lin- c-Kit+ Sca1+ CD48+ CD150-), which lost the ability of self-renewal, but develop into lineage-committed progenitors (common myeloid/lymphoid progenitors – CMP/CLP).

The granulocytic lineage commitment is marked by the differentiation of common myeloid progenitor (CMP) to granulocyte-monocyte progenitor (GMP), and the main driver of this transit is C/EBPα [6]. C/EBPα expression is maintained throughout granulocytic differentiation, forming dimers with other transcription factors (TF), and inhibiting *Myc* and thus halting progression through the cell cycle [7]. GMPs can give rise to both granulocytes and monocytes, therefore the granulocytic/monocytic production needs to be balanced. This is done by regulation of PU.1 level. C/EBPα in combination with low levels of PU.1 gives rise to granulocytes, while higher levels of PU.1 favor the production of monocytes [8]. Granulopoiesis is further enhanced by GFI-1, a TF that represses the transcription of both progenitor-specific and monocyte-specific genes and is necessary for granulocytic development as mice that are deficient for *Gfi1* are neutropenic [9]–[11].

The terminal maturation of granulocytes is marked by the declining proliferation potential and development of granules. The terminal stages of granulocytic development can be divided to the distinct proliferative stages: myeloblasts, promyelocytes, myelocytes and the non-proliferative stages: metamyelocytes, band cells and mature neutrophils [12].

C/EBPα is important in the early stages of terminal maturation, where it induces the expression of G-CSF-R and the formation of primary granules [13], [14]. Later on both C/EBPα and G-CSF-R activate C/EBPε which is necessary for terminal maturation and the production of secondary and tertiary granules [12], [14]–[16].

The observations mentioned earlier represent the so-called hierarchal model of hematopoiesis, this model suggests steps of gradual differentiation and the loss of ability to self-renewal while progressing down the developmental tree. However recently, with the introduction of single cell techniques it is becoming more evident, that this classical, hierarchal model is not a true representation of how hematopoietic development happens. In fact, the process is more of a continuum, much more complex with more types of progenitors for each lineage and cells with mixed lineage potential, as shown in Figure 1B [17], [18]. Contradictory to the hierarchal model of hematopoiesis, multiple groups have suggested that some lineage-bias is present already at the level of stem cells [19], [20], that HSC can differentiate into myeloid progenitors prior cell division [21] or that stem cells that are differentiation inactive are not quiescent [22]. Recently, it has also been shown that the MPP compartment, which was originally thought to be formed by multipotent cells, is actually consisting of 3 different lineage-biased subpopulations. These can be divided based on Flk2 expression: MPP2 and MPP3 have low Flk2 expression and mostly myeloid output, while MPP4, also referred to as lymphoid primed multipotent progenitors (LMPP), have high Flk2 expression and are biased towards lymphoid output [23]–[26].

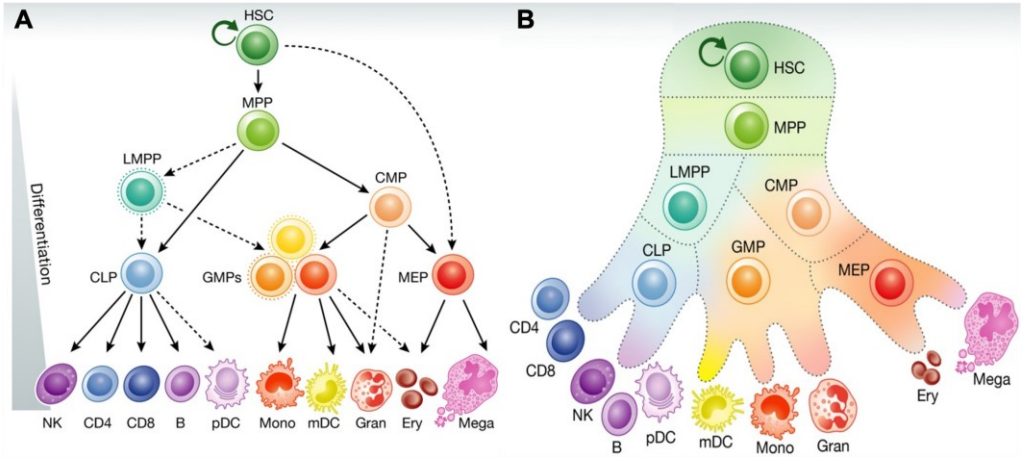


Figure 1. Models of hematopoiesis (picture adapted from [18]***).***
Two models of hematopoiesis. (A) hierarchal model which presents stepwise progression through the developmental tree with progressing differentiation and decreasing self-renewal potential. (B) Continuum model presenting the continuous development of cell types (pDC – plasmacytoid dendritic cells, mDC – myeloid DC).

Population diversity was shown also at the level of committed progenitors. Kwow *et al*. showed that the GMP compartment consist of at least 10 subpopulations. Among them they have identified a committed neutrophil progenitor proNeu1, that gives rise to proNeu2. proNeu2 develops into earlier identified preNeu, which is a direct progenitor of non-proliferating immature neutrophils. Interestingly, only proNeu1 population is expanded during sepsis at the expense of monocytes, suggesting differential roles for individual progenitor populations [27], [28].

3 Hematopoiesis under stress conditions

In steady state, HSCs maintain a balanced production of blood cells, however in stress conditions, like acute infection, bleeding, or recovery from irradiation or chemotherapy, HSCs must adjust cell production based on the needs of the organism.

Acute infection leads to the enhanced consumption of innate immune cells that are necessary to fight the pathogen, and therefore, cell production is shifted towards production of myeloid cells. That can be further dissected into three distinguished processes:

a) Emergency monocytopoiesis: This process is driven mostly by type one and two interferons (IFN) and ensures sufficient production of monocytes, macrophages and monocyte-derived DC during inflammation [29], [30].
b) Emergency megakaryopoiesis: Stress hematopoiesis also induces the proliferation of megakaryocytes, to rapidly make up for platelets that are used in big numbers during acute inflammation (thrombocytopenia) [31].
c) Emergency granulopoiesis (EG): And last, but not least, emergency granulopoiesis, which will be discussed in detail in this thesis.

4 Emergency granulopoiesis

Emergency granulopoiesis is defined as the enhanced and accelerated *de novo* production of granulocytes that happens as a reaction to infection in the organism [4]. Clinically, the signs of emergency hematopoiesis include high blood leukocytosis, neutrophilia, as well as the emergence of immature neutrophils in the blood (left-shift) [32]. EG can be dissected into 5 different stages [4], [32], [33]:

1) pathogen sensing
2) rapid release of granulocytes from the BM to circulation
3) expansion of myeloid progenitors
4) accelerated differentiation
5) termination of EG and re-establishment to SSG

Most studies focus on the first step of EG – pathogen sensing, making it the best characterized step during EG, while the other steps have been described mostly in the context of different infections and overall inflammatory state, and therefore the mechanisms might not be fully specific for EG.

The distinct steps of EG are characterized by activation of different TF that orchestrate the proper stepwise response. Surprisingly, the number of TF that have been reported as critical players in EG is rather limited. In this chapter we will review the individual steps necessary to carry out successful EG response and document the TFs mediating the switch between SSG and EG. Further, we will present the reported TF that regulate EG. Additionally, the mechanisms employed during EG in response to pathogens, are also employed during hematopoietic stress and recovery, caused by chemotherapy and irradiation, and therefore will also be documented in this thesis.

4.1 Pathogen sensing

EG is initiated by pathogen sensing, which can occur in one of two ways (Figure 2). The first option is mediated by mature immune cells or cells in the BM niche (endothelial and stromal cells). These cells sense the pathogen via TLR and produce cytokines to alert HSPCs in the bone marrow to induce EG [34], this is known as the indirect pathogen sensing. The second option – direct pathogen sensing, employs TLR on the surface of HSPCs that are able to sense the pathogen and initiate EG without cytokine stimulation [35], [36].

4.1.1 Direct pathogen sensing

HSPCs have TLRs on their surface, which allow them to sense pathogens directly. The TLRs that have been detected on the surface of HSPCs include: TLR2, TLR4, TLR7/8 and TLR9 [37]–[40]. The stimulation of these receptors leads to the activation of proliferation of the HSPCs and myeloid differentiation [37], [38], [41]. LPS is sensed by TLR4 and the downstream signaling can be mediated either by TRIF or MyD88 adaptor molecules. Repetitive treatment with low doses of LPS leads to the activation and proliferation of HSPCs, which is mediated by TRIF, downstream ROS production, and activation of p38 MAP Kinase. However, this pathway is dispensable for EG, as mice deficient in TRIF, as well as mice treated with ROS and p38 inhibitors do carry out EG, unlike MyD88 KO mice who fail to respond. Therefore MyD88 is the main signaling adaptor of TLR4 employed during EG [36].

Acute challenge with LPS induces changes in gene expression, as well as epigenetic changes in HSPCs 24 hours after the stimulation [42]. The gene expression changes are only transient and are not detected 4 weeks after the stimulation, unlike the epigenetic changes that persist and allow accelerated and enhanced response to the second challenge [42]. Zhao *et al*. had shown that TLR4 stimulation with LPS leads to activation of NF-κB in HSPCs and cytokine production at levels far greater than cytokine levels produced by mature myeloid or lymphoid cells. The cytokines, produced by HSPCs, mainly IL-6, were sufficient to induce myeloid differentiation. This suggests that stem cells are able to adapt hematopoiesis under stress conditions without stimulatory signals from other cell types [35].

4.1.2 Indirect pathogen sensing – cytokine regulation of EG

Pathogen sensing in HSPCs is also mediated by indirect mechanisms. WT animals transplanted with MyD88 KO HSPCs (therefore cannot sense LPS directly) do carry out EG response, whereas MyD88 KO animals transplanted with WT HSPCs do not [34]. This experiment indicates that the indirect sensing is the major and required pathway to initiate EG. The main cytokine that drives EG is G-CSF, which is produced in large quantities by niche endothelial cells during EG [34]. G-CSF binds to G-CSF-R, which activates STAT3, which translocates to the nucleus, and activates the expression of target genes [34], [43], [44].

G-CSF production by endothelial cells in the BM [34] during EG is dependent on Map3k8 expression in the endothelial cells, and mice deficient in Map3k8 have decreased levels of G-CSF and the animals cannot carry out a full EG response [45]. In line with that, constitutively active MAPK signaling leads to increased production of inflammatory cytokines, including all colony stimulating factors (CSFs), through NF-κB signaling and consequent myeloid biased blood and BM composition [46].

However, G-CSF or G-CSF-R deficient mice challenged with *Candida Albicans* do carry out EG [47]. And the same was observed using animals deficient in all three colony stimulating factors (G- , GM-, M-) when stimulated with thioglycate [48]. These suggest that there must be an alternative factor, that takes over inducing EG when these cytokines are missing.

One of the proposed factors is IL-6, since granulopoiesis is sustained by IL-6 production in G-CSF and GM-CSF deficient cells after LPS stimulation [49]. However, this seems to be in contradiction with observations by Basu *et al*. showing that mice deficient in IL-6 only or IL-6 and G-CSF do carry out EG following infection with *C. Albicans* [47]. Nevertheless, IL-6 deficient mice treated with 5-FU and LPS to induce de novo myelopoiesis showed insufficient production of Gr1+ Mac1+ cells [35]. Altogether, these observations might indicate that distinct pathogens may rely on distinct key cytokines to mediate EG.

To conclude, from the evidence presented here it seems that EG response is sustained on one hand by G-CSF, and partially by M-CSF and GM-CSF, produced by the BM niche, and on the other hand by IL-6 produced by stem cells and mature immune cells. However, what the relation between these factors is and how redundant they actually are and whether there are some other factors that may sustain EG in deficiency of all these factors is yet to be determined.

4.2 Release of granulocytes from the BM to blood

The retention versus mobilization of granulocytes is regulated by the antagonist effects of CXCL12/CXCR4 and CXCL2/CXCR2 and is regulated by G-CSF during inflammation [50], [51]. The initial rapid release of BM granulocytes is facilitated by increased levels of CXCR2 ligands (CXCL1 and CXCL2) produced by the BM niche in the acute phase of inflammation. In the later stages of inflammation G-CSF becomes the

main mobilizing agent by regulating the levels of both the retention factor CXCR4 and mobilizing factors CXCL1 and CXCL2 [51]–[53].

4.3 Expansion of myeloid progenitors

Once the signal has reached the HSPCs a distinct transcriptional program must be initiated to increase the output of granulocytes produced. First, the myeloid progenitor compartment has to be expanded. This is induced both by TLR receptor simulation [37], [41] and by stimulation with pro-inflammatory cytokines IL-1, IL-6, TNF-α, as well as IFNα and IFNγ [54]–[57]. This induces the expansion of two populations of myeloid progenitors: MPP2 that are megakaryocyte biased and MPP3 that are granulocyte-monocyte biased. At the same time, myeloid reprogramming of lymphoid biased MPP4 occurs, which leads to strong myeloid commitment, causing the output of the progenitors to be mostly myeloid cells during regeneration of the hematopoietic system following irradiation or 5-fluorouracyl (5-FU) treatment [26], [58], [59]. The increase in MPP2/3 populations was followed by the occurrence of compact GMP clusters (cGMP), that are not detectable in non-treated WT animals, but occur following depletion of granulocytes using Ly6G antibody, G-CSF treatment or during peak regeneration following 5-FU treatment or irradiation. This suggests that cGMP formation is characteristic for hematopoiesis during emergency conditions [58].

The myeloid output is caused mainly by the flooding of the system by factors stimulating myelopoiesis, however, additional factors may play a role as well. The favoring of granulopoiesis over lymphopoiesis is also carried out by regulation of growth and retention factors for lymphocytes produced by the stroma (such as SCF and CXCL12). During inflammation, high levels of G-CSF inhibit the production of these factors by the stromal cells to levels that suppress lymphopoiesis in the BM (Figure 2) [60], [61]. Lymphocytes are forced to mobilize to secondary lymphoid organs, hence freeing the space in the BM for granulocytic production. This seems to be induced by TNFα and IL-1β signaling [61], [62]. Additionally, ROS production by myeloid cells in the BM was also linked to progenitor expansion during EG [63].

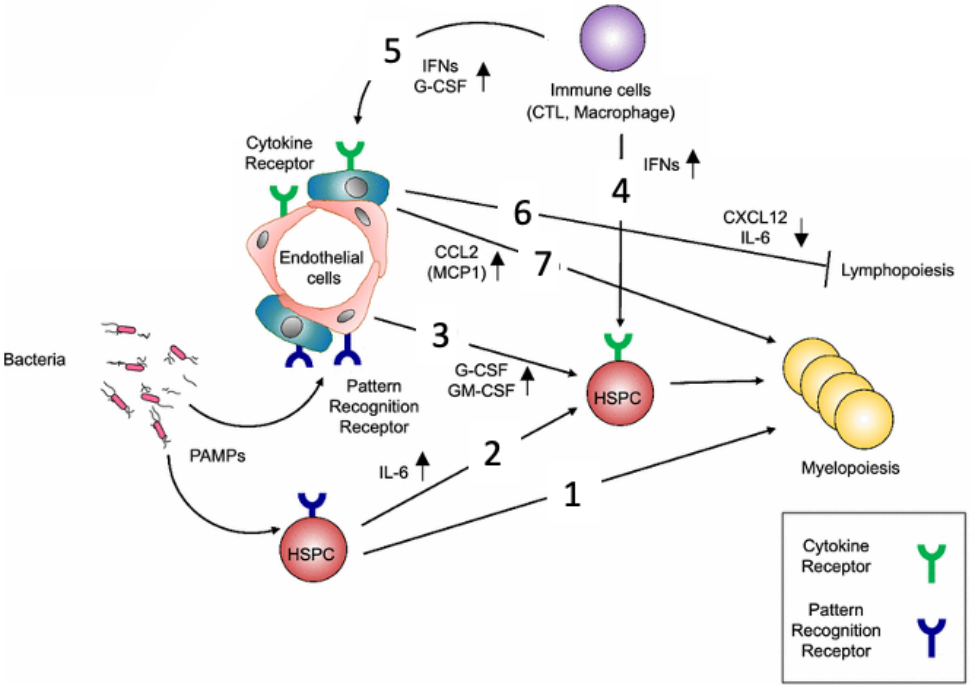


Figure 2. Induction of emergency response in HSPCs (adapted from [64]***)***
HSPCs sense the pathogen directly through PRRs and induce myelopoiesis (1) or produce IL-6 in paracrine manner (2). The pathogen can be sensed indirectly through PRR on surface of stromal and endothelial cells (3) or mature immune cells (4), which leads to production of CSFs and inflammatory cytokines to induce myelopoiesis in HSPCs. Endothelial cells are further stimulated by cytokines from mature immune cells (5) and produce factors that favor granulopoiesis over lymphopoiesis (6,7).

4.4 Accelerated differentiation of myeloid progenitors

The expansion of the myeloid progenitor compartment is followed by accelerated differentiation of the granulocyte precursors into granulocytes [65]. This is carried out by accelerated progression through the cell cycle which is orchestrated by C/EBPβ [66], [67].

4.4.1 Differential regulation of SSG vs EG by C/EBPα and C/EBPβ, respectively

C/EBPα and C/EBPβ are two members of the family of CCAAT/enhancer-binding proteins (C/EBPs), a family of leucine zipper transcription factors that are important for granulopoiesis regulation [68]. C/EBPα KO mice do not have any granulocytes present in the steady state, instead the granulocytic development is blocked at the CMP/GMP transition [6]. This can be overcome with IL-3 and GM-CSF stimulation *in vitro* and *in vivo*, but not G-CSF or IL-6, as C/EBPα KO diminishes expression of *Csfr3*, the gene coding for G-CSF-R, and greatly downregulates expression of *Il6r* [69]–[72]. Further,

C/EBPα slows down the cell cycle progression by inhibiting *Myc* and *Cdk2* and *Cdk4* (Figure 3A). This leads to arrest of progenitor proliferation and differentiation into granulocytes, thus ensuring homeostatic levels of granulocytes in the steady state [7], [73], [74].

Unlike C/EBPα KO, C/EBPβ KO mice have normal levels of granulocytes in steady state conditions, nevertheless, C/EBPβ KO mice are not able to carry out EG response [71], [75]. C/EBPβ drives EG by inducing entry to the cell cycle, progenitor proliferation and promoting differentiation into the myeloid lineage. This has been shown in the context of treatment with *C. Albicans* [66] and transplantation after lethal irradiation or 5-FU treatment [67]. In fact, out of all C/EBPs, only C/EBPβ mRNA levels increase following cytokine stimulation, and C/EBPβ protein level is also upregulated in HSPCs after 5-FU treatment [67], [71]. C/EBPβ is activated by G-CSF signaling through STAT3. Activation of C/EBPβ together with STAT3 leads to dissociation of C/EBPα from the *Myc* promotor, which allows the accelerated cell cycle progression and granulocytic differentiation (Figure 3B) [44], [66], [71].

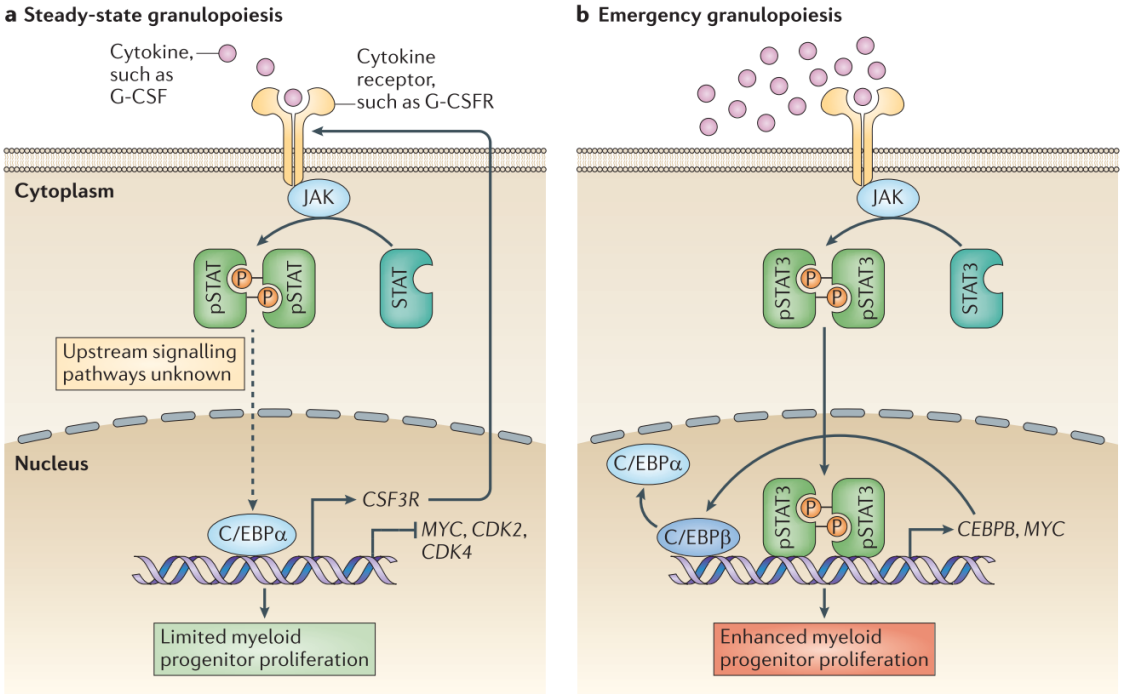


Figure 3. Differential regulation of steady state granulopoiesis (A) vs emergency granulopoiesis (B) by C/EBPα and C/EBPβ, respectively (picture from [4]**).**

Both steady state and emergency granulopoiesis are activated by G-CSF-R stimulation with G-CSF which activates downstream STAT3 signaling. In steady state C/EBPα inhibits expression of Myc and Cdk2 and 4 thus inhibiting progenitor proliferation. In emergency conditions C/EBPβ replaces C/EBPα and enhances the progenitor proliferation and granulocytic differentiation.

In conclusion, C/EBPα and C/EBPβ both drive granulopoiesis, but in different context, as shown in Figure 3. C/EBPα is the main driver of steady state granulopoiesis, while C/EBPβ is the main driver of emergency granulopoiesis.

4.4.2 Regulation of genomic stability during accelerated differentiation by FANCC

Fancc is a member of the Fanconi DNA repair pathway, which is crucial for DNA repair in the S phase. The deficiency in individual members of this pathway leads to Fanconi anemia, which is characterized by bone marrow failure [76]. *Fancc* expression has been shown to be activated by STAT3 and C/EBPβ in the early stages of EG and by IRF8 binding to a cis regulatory element of *Fancc* during the termination of EG [77], [78].

Indeed, *Fancc* expression is upregulated in GMPs following IL-1β or G-CSF stimulation and *Fancc* deficient mice are not able to carry out emergency granulopoiesis following alum injections. Repeated alum injections lead to occurrence of myeloid blasts, followed by bone marrow failure and death of the animals. *Fancc* deficient cells showed increased apoptosis in the stem cell, progenitor and granulocytic compartments during failed EG response. The increased apoptosis is initiated by TP53 after the detection of unrepaired replication fork and can be rescued by TP53 haploinsufficiency. However, the failed EG response is damaging to the HSPC as they are not able to reconstitute the myeloid compartment upon transplantation [77]–[79]. These data suggest that the expression of *Fancc* is an important mediator of genome stability, which is crucial for functionality of the cells as well as for the successful EG response, where new cells are produced at much higher and faster rates than in steady state.

4.5 Termination of EG and re-establishment to SSG

Once the pathogen has been cleared from the system, resolution phase has to be initiated. In this phase the apoptotic neutrophils must be cleared out by monocytes and macrophages and tissue healing must be initiated [80]. Apart from that, the emergency response must be terminated and granulopoiesis has to be switched back from emergency to steady state. In case this does not happen, the newly produced granulocytes are further infiltrating the tissues, causing tissue damage [81]. The unterminated expansion of the myeloid compartment might also lead to leukemia [82], [83]. The termination of the EG

response at the level of HSPCs is orchestrated by the decrease of inflammatory factors, such as IL-1 and IL-6, and the increase in factors inducing healing and quiescence, such as TGF-β and CXCL4. [58]

4.5.1 Regulation of EG termination by IRF8

IRF8 is a transcriptional factor expressed by myeloid progenitors and is suspected to play role in lineage determination. In steady state conditions the expression of IRF8 leads to repression of neutrophil development and promotes the development of DC, monocytes and macrophages. *In vivo* models demonstrated that IRF8 deficiency leads to elevated numbers of GMPs and increased production of granulocytes [84]–[86].

Mice deficient in *Irf8* do carry out EG, however the response is not terminated, and the granulocyte numbers do not return to baseline. Consequently, repeated episodes of EG in IRF8 KO mice leads to the development of leukemia and worsened survival of the animals [83]. In line with these observations, the levels of STAT3 are also increased significantly in *Irf8* deficient CD34+ cells following alum treatments and do not return to baseline, suggesting that IRF8 is necessary for termination of the EG response. One of the proposed mechanisms for the deregulation of the EG response in *Irf8* deficient mice is via β-catenin, and the control of proliferation and apoptosis by β-catenin target genes. Indeed, the expression of β-catenin and its target genes *Myc* and *Birc5* is increased and sustained in the progenitor cells from *Irf8* deficient mice during repeated treatment of EG that failed to be terminated [83].

4.6 Negative regulation of EG

EG is a complex process that must be regulated tightly. The factors known to play a role in the regulation have been described in the previous chapters. Other than that, there are two negative regulators that have been linked to EG. These limit the EG response to prevent tissue damage and exaggerated response.

SOCS3 is a negative regulator of G-CSF-R signaling that is activated by STAT3 and is crucial for regulation of EG response. Mice deficient in *Socs3* develop neutrophilia and suffer from chronic inflammation. The EG response in *Socs3* deficient animals upon G-CSF stimulation is marked by increased neutrophilia, followed by neutrophil infiltration to tissues and consequently to hind-leg paresis [44], [87].

Bcl3 is a member of the IκB family and one of the negative regulators of NF-κB signaling, which leads to the production of inflammatory cytokines during EG [46]. It is also activated by the G-CSF-R signaling, as two groups have independently identified STAT3 binding sites in *Bcl3* gene locus [88], [89]. *Bcl3* deficient mice stimulated with G-CSF accumulated twice as many granulocytes as WT controls. Ectopic expression of *Bcl3* and its target p50 in lineage depleted BM cells greatly attenuated granulocytic production, suggesting that *Bcl3* is negatively regulating granulocytic production during EG to prevent inflammation related injury [88]. In addition, Strauss *et al*. showed, that in the settings of carcinogenesis, inhibition of both *Bcl3* and *Socs3* by upregulation of RORC1 leads to tumor-promoting emergency granulo-monocytopoiesis which helps tumor growth and metastasis [90].

5 Canonical Wnt/β-catenin signaling pathway in the hematopoietic system

Wnt signaling is an evolutionary conserved signaling pathway that was first identified in drosophila and is one of the main developmental signaling pathways. Wnt signaling is involved in embryonic development, as well as the regulation of homeostasis and stem cell function in multiple tissues in adult organism [91].

The canonical signaling is activated by binding of Wnt ligands to a receptor which consists of the Frizzled receptors and LRP5/6 coreceptor. Up to date 19 Wnt ligands and 10 Frizzled receptors have been identified in mammals, however, how redundant these ligands and receptors are and how distinct and specific their function is, is poorly understood [92], [93]. The main signal transducer of canonical Wnt signaling is β-catenin. Deregulation of β-catenin and its activation has been linked to cancer development in multiple tissues [94]–[97]. When the signaling is inactive, β-catenin is constantly phosphorylated and degraded by the β-catenin destruction complex consisting of kinases GSK-3β and CK1, and anchor proteins AXIN1 or 2 and APC [98], [99]. Once Wnt ligands bind to the receptor, the destruction complex is inactivated and non-phosphorylated β-catenin accumulates and translocates to the nucleus, where it binds the TCF/LEF transcription mediating complex and modulates gene expression [100], [101].

Wnt signaling pathway and the role of β-catenin have been studied extensively in context of both healthy and diseased hematopoiesis, including acute myeloid leukemia and

chronic myelogenous leukemia [102], [103]. However, no consensus has been reached so far on the role that this pathway plays in the hematopoietic compartment. Both loss and gain of function at different levels of the pathway have been employed, yielding contradictory results [104].

Briefly, multiple groups using gain-of-function approaches have shown that activation of the Wnt signaling enhances HSC function, including proliferation, repopulation capacity and self-renewal [105]–[107]. The importance of Wnt signaling for proper HSC function was further supported by multiple loss-of-function approaches [103], [108], [109]. One the other hand, other studies employing inactivation of the pathway suggested that the pathway is fully dispensable for HSCs and that steady state hematopoiesis is unaffected by Wnt signaling deficiency [110]–[112]. In addition, some groups even showed that β-catenin activation might be harmful to the HSCs and that constitutive activation of Wnt signaling leads to multilineage differentiation block, exhaustion of the stem cell pool and consequently, death of the animals [113], [114].
The differences observed are usually explained by differences in modulation of the pathway, including differences in approaches, methods and especially modulation of the pathway at different levels, ranging from manipulation of extracellular signals, cytoplasmic proteins to nuclear factors. Additional explanation to differences observed with the gain-of-function approaches was provided by Luis *et al*. who suggested that these are caused by dosage dependent impact of Wnt signaling. Only mild Wnt signaling enhances HSC function, while higher levels of activation of the pathway impair HSC function [115].

To conclude, despite the many publications investigating Wnt/β-catenin signaling pathway in HSCs, its role remains controversial. Strikingly, the majority of these publications are dealing with hematopoiesis in the context of the steady state, and very little is known about the canonical Wnt signaling under stress conditions. Therefore, this topic will be explored in this thesis further.

5.1 Wnt/β-catenin signaling during hematopoietic stress

Most of the publications studying β-catenin in stress conditions employ the model of hematopoietic regeneration, this includes 5-FU treatment, Cyclophosphamide/G-CSF treatment (Cy/G) or γ-irradiation exposure. 5-FU and Cy are both chemotherapeutic drugs that deplete rapidly dividing hematopoietic cells, including mature cells and committed

progenitors, but not HSCs, which are instead induced to proliferate and repopulate the hematopoietic system and in case of the Cy/G treatment mobilize HSCs to the blood. Therefore, both drugs allow to study the capability of HSCs to regenerate the hematopoietic system [116], [117].

Activation of β-catenin signaling after 5-FU treatment has been observed by multiple groups. Congdon *et al*. reported increase in active β-catenin levels in LSK cells in WT mice upon 5-FU treatment. Similarly, employing a β-catenin reporter mouse (TCF H2B-GFP), Lento *et al*. showed upregulation of the GFP signal as early as 3 days following 5-FU treatment with a peak at 7 days, in both LSK and LK cells. Hérault at el. observed an increase in nuclear β-catenin in GMPs at day 8 following 5-FU treatment. In line with that, the β-catenin loss of function mice had significantly delayed formation of GMP clusters following the same treatment. Phenotypically, treatment of β-catenin null mice with 5-FU led to a decrease in total bone marrow cellularity, a decrease in progenitor counts, and a defect in generating mature cells, including lymphocytes, neutrophils and platelets. Further, repeated treatment with 5-FU led to decreased survival of β-catenin deficient mice [58], [118], [119].
Similar observations were reached using a model of irradiation and Cy/G treatment. In the Cy/G setting, the frequency of LSK cells positive for β-catenin increased significantly and it was accompanied by 2,8- and 3,6-fold increase in β-catenin target genes *Myc* and *Axin2*, respectively. Similarly, a 12-fold increase was observed in the frequency of β-catenin positive HSCs 14 days following irradiation (4.5 Gy), and mice with β-catenin deletion failed to recover and maintain the stem and progenitor cell compartment following irradiation [118], [119].

5.2 Wnt/β-catenin signaling during inflammation

In recent years, the canonical Wnt signaling pathway was shown to induce an inflammatory response in multiple tissues and cell types. For instance, it was reported that activation of β-catenin drives differentiation and proinflammatory functions in splenic dendritic cells following infection with *Toxoplasma Gondii* [120]. Similarly, Gong and colleagues showed that LPS stimulation leads to accumulation of cytosolic β-catenin and activation of migration in macrophages [121]. Further, activation of β-catenin in the context of acute lung injury was linked to enhanced lung inflammation by promotion of Th17 response, neutrophil infiltration and inflammatory cytokine

production [122], [123]. In this context, the knock down of β-catenin or inhibition of Wnt ligand production lead to diminished production of inflammatory cytokines following LPS treatment, possibly by a crosstalk between β-catenin and NF-κB signaling pathways [123]–[125]. In line with these observations, neutrophil transmigration through human epithelium was reported to also activate β-catenin in lung epithelial cells in inflammatory lung disease [126]. Altogether, there is increasing evidence that β-catenin signaling might be able to induce production of inflammatory cytokines, for example in human bronchial cells.

5.3 Wnt ligands in stress conditions

As mentioned previously, very little is known about the individual Wnt ligands and the role they play in site-specific β-catenin activation. Interestingly, both Congdon *et al*. and Lento *et al*. observed the production of Wnt10b in the BM following irradiation [118], [119]. The activation of Wnt10b was coinciding with the active β-catenin levels and HSC proliferation, suggesting that Wnt10b might be activating β-catenin signaling in HSPCs during stress conditions. In steady state, Wnt10b is produced by bone marrow cells, not the stroma [127]. However, following irradiation, the levels increase and Wnt10b is produced mostly by endothelial cells, with a smaller fraction of hematopoietic cells also producing Wnt10b [118], [119], [127]. Indeed, immune cells in the BM produce low levels of Wnt10b, which is able to induce activation of Wnt signaling and activate target gene activation in MPPs [128], [129]

Increase in Wnt ligands has been observed also during infections caused by different pathogens, as reviewed previously [130]. Interestingly, the Wnt ligand production and β-catenin activation was correlated to production of inflammatory cytokines in both human sepsis and a mouse model of endotoxemia. In the mouse model, Wnt10b showed the biggest increase that correlated with the increase in β-catenin itself and *Wisp1*, a β-catenin target gene. In human sepsis WNT10B showed a positive correlation with IL-6 and TNF levels. Remarkably, Wnt10b production was dependent on TLR4-MyD88 signaling, as the increase of Wnt10b following LPS treatment was only detectable in *Trif* KO mice, but not in *Myd88* deficient mice. Interestingly, when the mice were pre-treated with a β-catenin signaling inhibitor, the LPS-induced proinflammatory cytokine response was impaired [131]. Accordingly, inhibition of β-catenin signaling in the model of mouse

sepsis caused by cecal ligation and puncture led to decreased plasma levels of both IL-6 and TNF as well as the overall lung inflammation and neutrophil infiltration [132].

All the evidence presented here is suggesting that (1) inflammatory conditions can lead to activation of the Wnt/β-catenin signaling pathway, and that (2) β-catenin is able to elicit an inflammatory response and might be important in production of inflammatory mediators. Remarkably, the activation of the pathway is necessary for successful regeneration of the hematopoietic system after chemotherapy or irradiation. However very little is known about β-catenin levels and mechanisms of activation at the level of HSPCs during inflammatory conditions like EG.

6 Aims of the study

EG is a crucial process involved in inflammatory response, pathogen clearance and recovery of the hematopoietic compartment. The process has been described at the level of effector cells (neutrophils fighting the infection) and progenitor populations (mainly GMPs). However, very little is known about the early changes that occur in HSPCs during an acute infection. We hypothesize that the changes taking place in HSPCs right upon infection are crucial to initiate and to successfully accomplish EG. In this thesis we aim to elucidate those critical changes in HSPCs. To test our hypothesis and identify critical changes occurring in HSPCs during EG, we designed 2 aims:

1. **To determine whether β-catenin gets activated in HSPCs during EG.**
   Since our previous work showed that β-catenin might be important for both steady state and emergency granulopoiesis, we will center this first aim on this pathway. This aim will be divided in two sub-aims:
   a. **To validate a murine model to investigate the role of canonical Wnt signaling pathway in steady state and emergency granulopoiesis**
      In this sub-aim we will validate a murine model characterized by inactivation of the β-catenin-TCF/LEF transcription mediating complex and analyze the impact of disruption of β-catenin signaling in the granulocytic compartment and EG response.
   b. **To determine whether active β-catenin gets accumulated in HSPCs during EG**
      In this aim we will determine whether β-catenin gets activated in HSPC *in vivo* following LPS stimulation. We will also assess this *in vitro* to determine whether this activation is direct or rather caused by the overall inflammatory state induced in the animal. The *in vitro* settings will also allow us to investigate the dynamics of the activation.
2. **To investigate early changes that happen at the level of HSPCs during EG.**
   In this aim we will perform scRNA-seq analysis at an early timepoint of EG that we will determine. This analysis will allow us to elucidate the changes in the composition and gene expression of the HSPC compartment that occur following LPS treatment.

7 Materials and Methods

7.1 Materials

7.1.1 Solutions, buffers and chemicals

Chemicals

- Chloroform (Sigma-Aldrich®, USA)
- Ethanol (Lach-Ner, s.r.o., Czech Republic)
- Methanol (Lach-Ner, s.r.o., Czech Republic)
- Pierce™ 16% Formaldehyde (w/v), Methanol-free (Thermo Fisher Scientific, USA)
- Propanol (Lach-Ner, s.r.o., Czech Republic)
- TRI Reagent® (Molecular Research Center, Inc., USA)

Buffers and media

- ACK lysis buffer (150mM NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA)
- Bovine serum albumin (BSA) (Sigma-Aldrich®, USA)
- Gibco Fetal bovine serum (FBS) (Thermo Fisher Scientific, USA)
- IMDM (Media, IMG)
- MACS buffer (PBS, 0.5% BSA, 2 mM EDTA, Media, IMG)
- Methocult GF M3434 (Stemcell Technologies, Canada)
- Phosphate buffered saline (PBS) (Media, IMG)

Enzymes and carriers

- Ambion™ DNase I (RNase-free) (Invitrogen™, USA)
- Anti-biotin MicroBeads ultrapure (Miltenyi Biotec GmbH, Germany)
- Glycogen RNA grade (Thermo Fisher Scientific, USA)
- LightCycler® 480 SYBR Green I Master (Roche Molecular Systems, USA)
- SuperScript™ II Reverse Transcriptase (Thermo Fisher Scientific, USA)

Cytokines and stimulants

- CHIR99021 (Sigma-Aldrich®, USA)
- Cytokines: murine SCF, IL-3, human IL-6 (PeproTech, USA)
- Pam3CSK4 (InvivoGen, USA)
- Recombinant Mouse Wnt-10b Protein (R&D Systems, Inc, USA)
- Ultrapure LPS from E. coli 0111:B4 (InvivoGen, USA)

7.1.2 Antibodies

Table 1. List of antibodies used in this thesis

Target	Fluorophore	Clone	Manufacturer
Anti-rabbit IgG (H+L)	AF647	-	Life technologies, USA
B220	Bio	RA3-6B2	BioLegend®, Inc., USA
c-Kit	Bio	2B8	BioLegend®, Inc., USA
c-Kit	PE	2B8	BioLegend®, Inc., USA
Cd11b	APC	M1/70	BioLegend®, Inc., USA
Cd11b	Bio	M1/70	BioLegend®, Inc., USA
CD150	PECy7	TC15-12F12.2	BioLegend®, Inc., USA
CD16/32	PECy7	93	BioLegend®, Inc., USA
CD3e	Bio	145-2C11	BioLegend®, Inc., USA
CD34	AF700	MEC14.7	BioLegend®, Inc., USA
CD48	FITC	HM48-1	BioLegend®, Inc., USA
Dead cells	Hoechst 33258	-	Sigma-Aldrich®, USA
Gr1	PE	RB6-8C5	BioLegend®, Inc., USA
Gr1	Bio	RB6-8C5	BioLegend®, Inc., USA
Lineage	PB	-	BioLegend®, Inc., USA
Non-phospho β-Catenin (Ser33/37/Thr41)	-	D13A1	Cell Signaling Technology, USA
Sca-1	FITC	D7	BioLegend®, Inc., USA
Sca-1	APC	D7	eBioscience®, USA
Streptavidin	eFluor450	-	eBioscience®, USA
Ter119	Bio	TER-119	BioLegend®, Inc., USA

7.1.3 qPCR primers

Table 2. List of qPCR primers used in this thesis

Gene	Forward primer 5' – 3'	Reverse primer 5' – 3'
Actb	GATCTGGCACCACACCTTCT	GGGGTGTTGAAGGTCTCAAA
Axin2	GATGTCTGGCAGTGGATGTTAG	GACTCCAATGGGTAGCTCTTTC
Bcl3	GTGGATGAGGATGGAGACA	AGGCTGAGTATTCGGTAGAC
Cebpb	AAGCTGAGCGACGAGTACAAGA	GTCAGCTCCAGCACCTTGTG
Cebpe	AAGGCCAAGAGGCGCATT	CGCTCGTTTTCAGCCATGTA
Ctsg	CTGACTAAGCAACGGTTCTGG	GATTGTAATCAGGATGGCGG
Ela2	ACTCTGGCTGCCATGCTACT	GCCACCAACAATCTCTGA
Gapdh	AACTTTGGCATTGTGGAAGG	ATCCACAGTCTTCTGGGTGG
Il6	GACCTGTCTATACCACTTCA	GCATCATCGTTGTTCATA
Lef1	GCTGCCTACATCTGAAACATGGTG	CTGTGGAGACAGTCTGGGG
Ltf	TATTTCTTGAGGCCCTTGGA	TCTCATCTCGTTCTGCCACC
Mpo	GGAAGGAGACCTAGAGG	TAGCACAGGAAGGCCAAT
Mpp9	ACGGTTGGTACTGGAAGTTCC	ACGGTTGGTACTGGAAGTTCC
Nkd1	AGGACGACTTCCCCCTAGAA	TGCAGCAAGCTGGTAATGTC
Tcf7	GCCAGAAGCAAGGAGTTCAC	TACACCAGATCCCAGCATCA
Tcf7l1	CCCCCTACTTTCCCAGCTAC	CTTTGTGTTTCCCCCTTCCT
Tcf7l2	CGTAGACCCCAAAACAGGAAT	TCCTGTCGTGATTGGGTACA
Wnt10b	GTTCTCTCGGGATTTCTTG	CACTTCCGCTTCAGGTTT

7.1.4 Kits and sets

- Chromium next germ single cell 3' reagent kit (10X Genomics, USA)
- G-CSF Quantikine ELISA Kit (R&D Systems, USA)
- RNeasy Mini Kit (QIAGEN, Germany)

7.1.5 Disposables and other materials

- 1,5 ml centrifuge tubes (Eppendorf AG, Germany)
- 15 ml, 50 ml falcon tubes (Techno Plastic Products®, Switzerland)
- Cell culture plates (12 well) (Techno Plastic Products® AG, Switzerland)
- Goldenrod Animal Lancet (MEDIpoint, Inc., USA)
- LightCycler® 480 Multiwell Plate 384, white (Roche Molecular Systems, USA)
- Multiwell plate, polypropylene 96-well V (Sigma-Aldrich®, USA)
- Needles 0.3×12 mm (Sterican®, Germany)
- Syringes 1 ml, 5 ml (Inject® Solo, Germany)

7.1.6 Instruments

- Cell counter machines: Cellometer® Auto T4 Plus Cell Counter (Nexcelom Bioscience, USA), TC20 Cell counter (Bio-Rad, USA),
- Centrifuges: Centrifuge 5417 R, Centrifuge 5810 R (Eppendorf AG, Germany)
- Cyclers: T100 Thermal Cycler (Bio-Rad, USA); LightCycler® 480II Real-Time PCR Systems (Roche Molecular Systems, USA)
- Elisa plate reader: Tecan Infinite M200 (Tecan, Switzerland)
- Flow cytometers and sorters: FACSymphony™ A5, BD Influx ™ Cell Sorter (BD™ Biosciences, USA)
- Incubator: MITRE 4000 Series, (Contherm Scientific Limited, New Zealand)
- Magnetic separator: autoMACS® Pro Separator (Miltenyi Biotec GmbH, Germany)
- Nanodrop: NanoDrop® 2000 Spectrophotometer (Thermo Scientific™, USA)
- scRNA-seq: Chromium controller instrument (10X Genomics, USA); Agilent 2100 Bioanalyzer (Agilent, USA); NextSeq® 500 (Illumina, USA)
- Vortex: IKA® Vortex 3 (IKA, Germany)

7.2 Methods

7.2.1 Animal handling and processing

Mouse handling

All mice used for experiments were males between 8 and 14 weeks old (except for animals used for the aging experiment, in that case males of ages 14, 20, 38 and 51 weeks were used). We employed C57Bl6/J mice (referred to as WT), and transgenic dnTCF4 mice crossed to VAV-iCRE. Mice were housed under SPF conditions in the IMG Animal Facility in Krč. All experiments with mice were performed under institutional approval.

LPS treatment

Mice were injected intraperitoneally with 35 µg of LPS and sacrificed at various timepoints following LPS injection (2, 4, 6, 12, 24 hours). Alternatively, for greater response, mice were given one additional injection, 48 hours after the first one. These mice were sacrificed 72 hours after the first injection. Blood and bone marrow cells were collected and analyzed.

BM cell isolation

Mice were sacrificed by cervical dislocation and legs washed with 70% ethanol. Tibia, femur and hip bones were isolated, cleaned from tissue and transferred to PBS 2% FBS on ice. The bones were crunched in 10 ml PBS 2% FBS and supernatant was collected into 15 ml falcon tubes on ice and spun down (450 g, 5 minutes, 4 °C). The supernatant was discarded, and the cell pellet was resuspended in 3 ml ACK lysis buffer for 5 minutes at room temperature to lyse erythrocytes. ACK was then diluted with 12 ml of PBS 2% FBS to stop the lysis and cells were spun again (450 g, 5 minutes, 4 °C). Cells were resuspended in 2 ml PBS 2% and filtered through nylon mesh into a new falcon tube, counted and resuspended in adequate volume.

Blood cell isolation

Approximately 100 µl of blood was collected from mouse retro-orbital vein into 1,5 ml eppendorf tube with 10 µl of heparin to prevent coagulation. 50 µl of blood was transferred into 15 ml falcon tube. The blood was lysed with 3 ml ACK for 5 minutes at room temperature, then washed with 12 ml of PBS 2% FBS and spun (450 g, 5 minutes, 4 °C). Supernatant was removed and the cells were lysed with additional 1 ml of ACK for 5 minutes, washed with 5 ml PBS 2% FBS and spun (450 g, 5 minutes, 4 °C).

Supernatant was discarded and the pellet was resuspended in the leftover volume and stained for FACS.

Blood serum collection and G-CSF level assessment by ELISA

Peripheral blood was collected from retro-orbital vein of PBS/LPS injected mice and left for 30 minutes undisturbed at room temperature to coagulate. The clot was removed by centrifugation (2300 g, 10 min, 4°C). The supernatant was transferred into a new tube and centrifuged again (1300 g, 10 min, 4°C). The supernatant was transferred into a new tube one more time and the samples were frozen by liquid nitrogen. G-CSF levels were assessed by Mouse G-CSF Quantikine ELISA Kit according to manufacturer's instruction.

7.2.2 FACS and sort

FACS staining for extracellular markers

Cells were counted and the desired cell count was plated into a 96 well FACS plate. The cells were spun down (450 g, 5 minutes, 4 °C) and the supernatant was discarded. Cells were resuspended in 50 µl of staining mix and stained for 30-45 minutes in the fridge. Then the cells were washed with 150 µl PBS 2% FBS and spun (450 g, 5 minutes, 4 °C). Cells were resuspended in 70 µl PBS 2% FBS and measured on FACS Symphony. FACS data was analyzed in FlowJo™ 10.6.2 (BD™ Biosciences, USA) and populations were gated as shown in Figure 4.

FACS staining for intracellular β-catenin

10^6 cells were plated per well in FACS plate and spun (450 g, 5 minutes, 4 °C). The supernatant was discarded, and cells were resuspended in 100 µl of staining mix to stain for extracellular markers and stained in the fridge for 30 minutes. The cells were washed with 100 µl PBS 2% FBS and spun (450 g, 5 minutes, 4 °C). Supernatant was discarded and the cells were resuspended in 200 µl ice-cold PBS and 63 µl of 16% formaldehyde and incubated in the fridge for 30 minutes to fix the cells. After that, the cells were spun (1300 g, 3 minutes, 4 °C) and the supernatant was discarded. The cells were then resuspended in 70 µl ice-cold 90% methanol and incubated in the freezer (-20°C) for 30 minutes to permeabilize the cells. After that the cells were washed twice – first with 150 µl and then with 200 µl PBS 0,5% BSA and spun down every time (1300 g, 3 minutes, 4 °C). Cells were then resuspended in 50 µl of staining mix (Anti-β-catenin antibody, dilution 1:100) and incubated in the fridge overnight. The cells were washed twice the

next day – first with 150 µl and then with 200 µl PBS 0,5% BSA and spun down every time (1300 g, 3 minutes, 4 °C). Next, the anti-rabbit IgG was added (1:500 dilution, in 100 µl PBS 0,5% BSA) and incubated for 30 minutes in the fridge. The cells were then washed twice again, spun down and resuspended in 80 µl PBS and measured on FACS Symphony. FACS data were analyzed in FlowJo and active β-catenin levels were determined by Mean fluorescence intensity (MFI) and visualized by heatmaps and histograms. To be able to analyze replicate experiments together, data were analyzed and graphed as fold increase from untreated samples.

LSK/HSC/MPP sort

Bone marrow cells were isolated as described above and lineage depletion was done to remove the fraction of mature cells from the bone marrow. Briefly, the cells were stained with 12 µl of staining mix containing biotinylated antibodies targeting markers of mature immune cells (B220, Ter119, CD3e, Gr1, Cd11b) and incubated for 30 minutes in the fridge. Cells were then washed with 10 ml MACS buffer and spun down (450 g, 5 minutes, 4 °C). Next, 20 µl of anti-biotin microbeads was added, vortex thoroughly and incubated for 20 minutes in the fridge. The cells were washed with 10 ml MACS buffer and spun down (450 g, 5 minutes, 4 °C). The pellet was resuspended with 2 ml MACS buffer and filtered through nylon mesh into a new 15 ml falcon tube. The cells were then separated on MACS sorter using the program DEPLETE_S. The negative fraction was spun down (450 g, 5 minutes, 4 °C) and stained with 200 µl of sort staining mix (extracellular markers of sorted population) and incubated for 30 minutes. Then the cells were washed, spun down (450 g, 5 minutes, 4 °C) and the supernatant was discarded. The pellet was cracked, the cells were filtered and diluted in HBSS and stained with Hoechst 33258 for cell viability prior to sorting. Target populations (LSKs, MPPs and HSCs) were sorted into 500 µl PBS 2% FBS using two-way sorting on the Influx instrument.

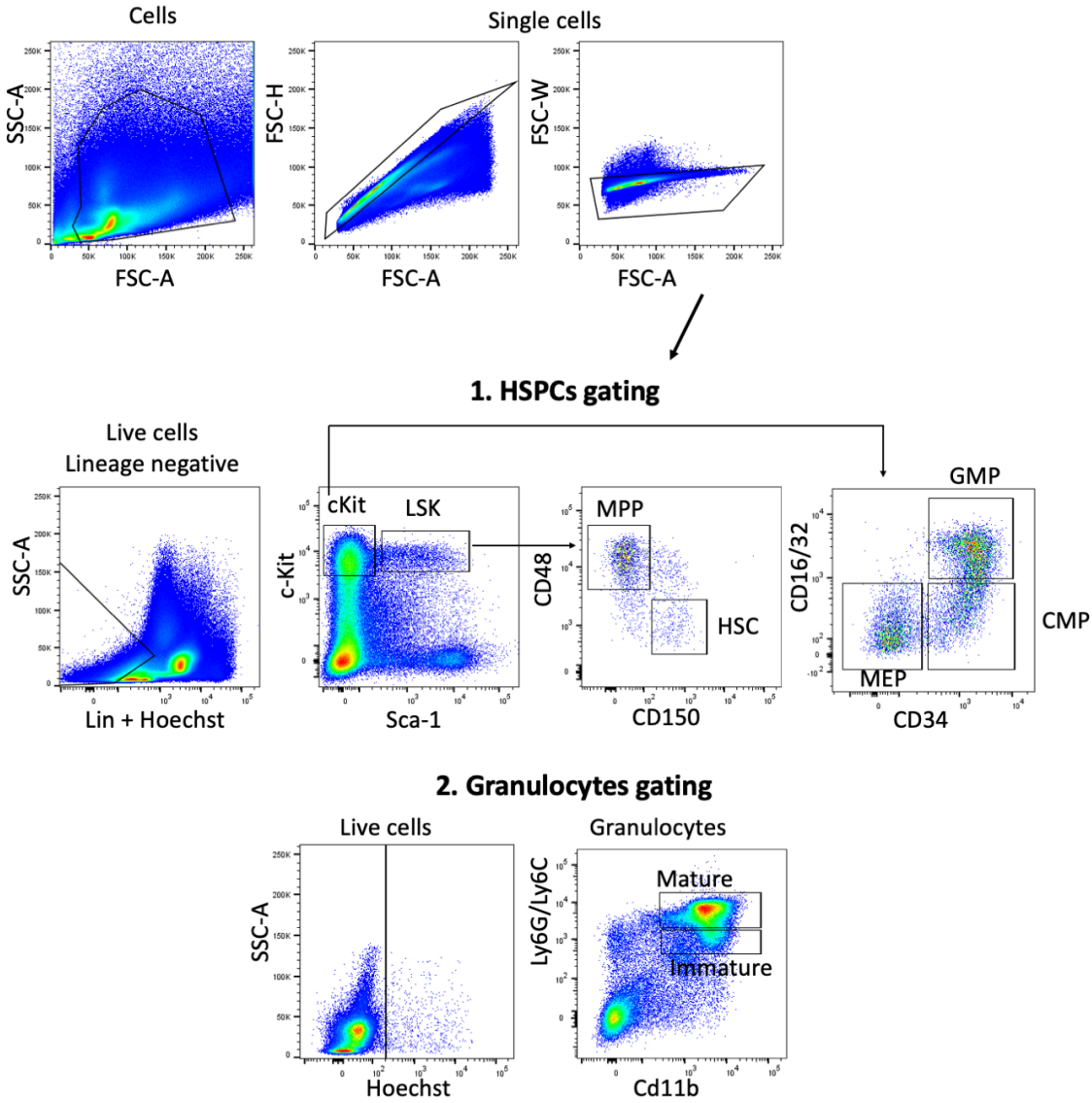


Figure 4. Gating strategy employed to analyze distinct BM populations.

7.2.3 Cell culture

c-Kit enrichment and culture conditions

Mice were sacrificed and BM cells isolated as described above. Cells were resuspended in 400 µl PBS 2% FBS and 4 µl of biotinylated anti c-Kit antibody was added. Cells were stained in the fridge for 25 minutes, washed with 10 ml MACS buffer and spun (450 g, 5 minutes, 4 °C). Then, 15 µl of anti-biotin microbeads was added into the dead volume and incubated for 20 minutes, washed with 10 ml MACS buffer, spun and then filtered with 2 ml MACS buffer into a new 15 ml falcon tube. Cells were then magnetically separated on MACS, using POSEL_S program (positive selection). Sorted cells (positive fraction) were counted and plated in 12 well plate at concentration 2 x 10^6 cells per 1 ml of c-Kit cultivation media (Table 3). Cells were incubated for 2 hours at 37 °C before

stimulants were added. Cells were collected at various timepoints of stimulation and spun down.

Table 3. c-Kit+ cell cultivation media composition and stimulus

Supplement	Concentration
IMDM	-
FBS	15%
ATB	1%
mSCF	100 ng/ml
mIL-3	20 ng/ml
hIL-6	20 ng/ml

Stimulus	Concentration
CHIR99021	10 mM
LPS	100 ng/ml
Pam3CSK4	1 ug/ml
Wnt10b	100 ng/ml

Colony culture and re-plating assay

3 x 10^4 cells in 135 µl IMDM + 2% FBS were added into 1350 µl of Methocult GF M3434 semi-solid medium with 1% ATB and mixed with a needle and syringe multiple times. Next, 500 µl of the cell and medium mixture was plated using the syringe and needle into 24 well plate in duplicates and incubated at 37 °C. The cells were collected after 7 days by washing the medium with 3 + 3 ml of PBS 2% FBS, spinning down (450 g, 5 min, 4 °C) and washing with additional 5 ml of PBS 2%FBS.

7.2.4 Gene expression analysis

RNA isolation using Trizol

RNA was isolated using combination of Trizol and Fenol-chloroform isolation, in case of lower cell counts RNAeasy mini kit (Qiagen) was used.

Cells were lyzed in 1 ml of Trizol and kept frozen at -80 C. After defrosting, 200 µl of chloroform was added and tubes were shaken for 20 second, then spun down (12 000 g, 15 minutes, 4 °C). Then, the top, transparent phase, was transferred into a new eppendorf tube with 570 µl isopropanol and 1 µl of RNA grade glycogen. The sample was shaken and spun again (12 000 g, 5 minutes, 4 °C). Supernatant was removed and the pellet was washed with 1 ml 75% ethanol and spun again (12 000 g, 5 minutes, 4 °C). The supernatant was removed thoroughly, and the tubes were left open for 30 second to allow the leftover ethanol to evaporate. The pellet was resuspended in 8,5 µl RNA grade water. The RNA concentration and quality were analyzed on Nanodrop machine.

Dnase treatment

Dnase treatment was applied in order to eliminate genomic DNA contamination from RNA. All samples were brought to the same concentration (up to 2 mg RNA in 8,5 µl water), next, 1 µl of Dnase buffer and 0,5 µl of Dnase were added per sample and the samples were incubated at 37 °C for 30 minutes. The reaction was terminated by adding 1 µl of EDTA and incubating at 70 °C for 10 minutes.

Reverse transcription

1 µl of random primers (200 ng/µl) and 1 µl of dNTPs (10 mM each) were added to each sample and samples were incubated at 65 °C for 5 minutes. Next, 4 µl 5xFS buffer and 2 µl DTT were added together with 1 µl with SuperScript II reverse transcriptase and incubated in PCR cycler at 25 °C for 10 minutes, followed by 42 °C for 50 minutes and 70 °C for 15 minutes. cDNA was stored at -20 °C.

Real Time qPCR

Table 4. Composition of qPCR reaction

Component	Per well
Sybr green I master mix	2,5 µl
Primer mix F+R (5 µM)	0,5 µl
cDNA	2 µl

cDNA was diluted at least 4 times and the reaction was set up as shown in Table 4 with primers listed in Table 2 and run on Roche Light Cycler 480 II. All samples were analyzed in duplicates. The geometric mean of *Actin* or *Gapdh* CT values was used for normalization of measured CT values. The data were presented as fold increase to be able to present replicate experiments.

Single cell RNAseq (scRNA-seq)

10 C57Bl6/J mice (10-12 weeks old) were injected with LPS as described above (1 injection) and sacrificed 4 hours after the injection. Additional 10 C57Bl6/J were injected with PBS as a control and sacrificed 4H later. Blood and bone marrow cells were isolated and processed and granulocytic response was assessed by cell counts and FACS analysis. MPPs and HSCs were sorted as described earlier. Next, 20 000 cells were pooled for each sample and viability was assessed by trypan blue in TC20 cell counter. Single cell RNA sequencing libraries were prepared using Chromium next germ single cell 3' reagent kit on Chromium controller instrument with a target of 4 000 cells per sample. The libraries were analyzed using Agilent 2100 Bioanalyzer and sequenced in two runs of NextSeq 500 sequencer with NextSeq 500/550 high output kit (75 cycles).

scRNA-seq analysis

Cell ranger pipeline was employed to process the raw data and to generate a matrix of unique molecular identifier (UMI) counts and the matrixes were analyzed in the R/Bioconductor statistical environment. Empty droplets were removed, and cell filtering was performed using defined standard parameters (UMI 1 000-50 000, minimum of 1 000 genes detected, 20% maximum mitochondrial genes expression, minimum 10% ribosomal gene expression). The estimated number of cells with mean reads and median genes per cell can be found in Table 5. Post-filtering QC was performed, and data were normalized by deconvolution, reduced in terms of dimensionality using uniform manifold approximation and projection (UMAP) on highly variable genes, clustered (graph-based clustering, consensus clustering - SC3, or other suitable method) and projected for interpretation. Gene set enrichment analysis (GSEA) was performed to identify pathways enriched in individual clusters that were identified in the scRNA sequencing. GO datasets were used as well as custom geneset based on signature genes for MPP2, MMP3 and MPP4, published by Pietras *et al.* [26]

Table 5. Number of cells and genes identified using scRNA-seq

Sample	Estimated number of cells	Mean reads per cells	Median genes per cell
HSC PBS	3663	64888	3628
HSC LPS	3539	65393	3346
MPP PBS	3098	67059	4265
MPP LPS	3716	61719	4046

7.2.5 Statistical analysis

Data were graphed and analysis was carried out in GraphPad Prism® 5.0 software (GraphPad Software, USA). Statistical significance was determined using unpaired 2-tailed Student t test. P values < 0,05 were considered statistically significant: $p < 0,05$ (*), $p < 0,01$ (**) and $p < 0,001$ (***).

In the GSEA analysis pathways with false discovery rate (FDR) < 0,25 were considered statistically significant and were presented in heatmap based on their normalized enrichment score (NES), the non-significant geneset were assigned with NES = 0.

8 Results

8.1 Introduction of dnTCF4 into murine hematopoietic cells impairs granulopoiesis

Transcriptional regulation of β-catenin target genes is exerted by TCF/LEF factors. As shown in Figure 5A, when β-catenin gets degraded in the cytoplasm, the TCF/LEF factors are inactivated by Groucho complex, preventing β-catenin dependent transcription. Once β-catenin gets stabilized and translocates to the nucleus, it replaces Groucho, binds to TCF/LEF factors and activates transcription. We have employed a transgenic mouse model which expresses a truncated form of human TCF4 (dnTCF4) transcription factor and has been crossed to Vav-iCre, therefore expressing the dnTCF4 transgene in the hematopoietic compartment. Unlike WT TCF4, dnTCF4 lacks the β-catenin binding domain and therefore abrogates the β-catenin mediated transcription, without disrupting other functions β-catenin may have outside binding TCF/LEF factors [133].

Four TCF/LEF genes have been identified in the genomes of vertebrate: *TCF7, TCF7L1, TCF7L2* and *LEF1*, encoding for TCF1, TCF3, TCF4 and LEF1 proteins, respectively [101]. First, we assessed the expression of these factors in murine whole bone marrow of differently aged animals to determine whether age-dependent changes in expression could drive an age-dependent phenotype. Nevertheless, no major changes in expression of neither of these TF were observed with increased age (Figure 5B).

To determine whether the introduction of dnTCF4 transgene into murine hematopoietic compartment abrogates β-catenin mediated gene expression, we cultivated c-Kit+ cells from BM of WT (grey) or dnTCF4 (red) mice in the presence of CHIR99021 for indicated timepoints. CHIR99021 is an activator of β-catenin signaling. It exerts its function by inhibiting GSK-3β and therefore preventing degradation of β-catenin. Cultivation of c-Kit+ cells with CHIR led to increase in expression of two β-catenin target genes, *Axin2* and *Nkd1,* in WT cells. However, this increase was not detectable in dnTCF4 cells cultured under the same conditions (Figure 5C), indicating that introduction of dnTCF4 was sufficient to block β-catenin-TCF/LEF mediated transcription.

Next, to analyze the impact of dnTCF4 transgene on granulopoiesis, cells were cultured in semi-solid medium favoring the differentiation of granulocytic/monocytic cells and

RT-qPCR analysis of granulocytic markers that are predominant in immature granulocytes or mature granulocytes was carried out (Figure 5D). The dnTCF4 cells had increased mRNA levels of immature granulocyte markers, namely neutrophil elastase (*Ela2)* and Cathepsin G (*Ctsg)* and reversely decreased levels of *Cebpe* and Lactoferin (*Ltf*), which are highly expressed in mature granulocytes.

Next, we investigated the impact of dnTCF4 on EG response. We induced EG in WT and dnTCF4 animals with 2 LPS injections as described before by Boettcher *et al*. [34], depicted in Figure 5E. We analyzed the response of neutrophils 72 hours after the first injection (Figure 5F). dnTCF4 have initially lower levels of neutrophils in blood and BM and once challenged with LPS are not able to increase neutrophilic levels in blood to WT levels. Analysis of BM neutrophils showed no differences in mature neutrophils, however, dnTCF4 BM had significantly decreased levels of immature neutrophils, after LPS challenge. To determine what caused the decreased production of neutrophils, we analyzed the BM progenitor compartment (Figure 5G). In the steady state, dnTCF4 had significantly increased percentage of CMPs and the increase was still detectable after LPS challenge, reversely, the percentage of GMPs was significantly reduced following the LPS treatment, suggesting a block at the level of granulocytic progenitors. Since both granulocytic mobilization and differentiation from progenitors is dependent on G-CSF, we assessed G-CSF levels in serum of PBS or LPS treated animals (Figure 5H). However, we did not detect any difference in the serum G-CSF levels between WT and dnTCF4 animals in neither steady state nor emergency conditions.

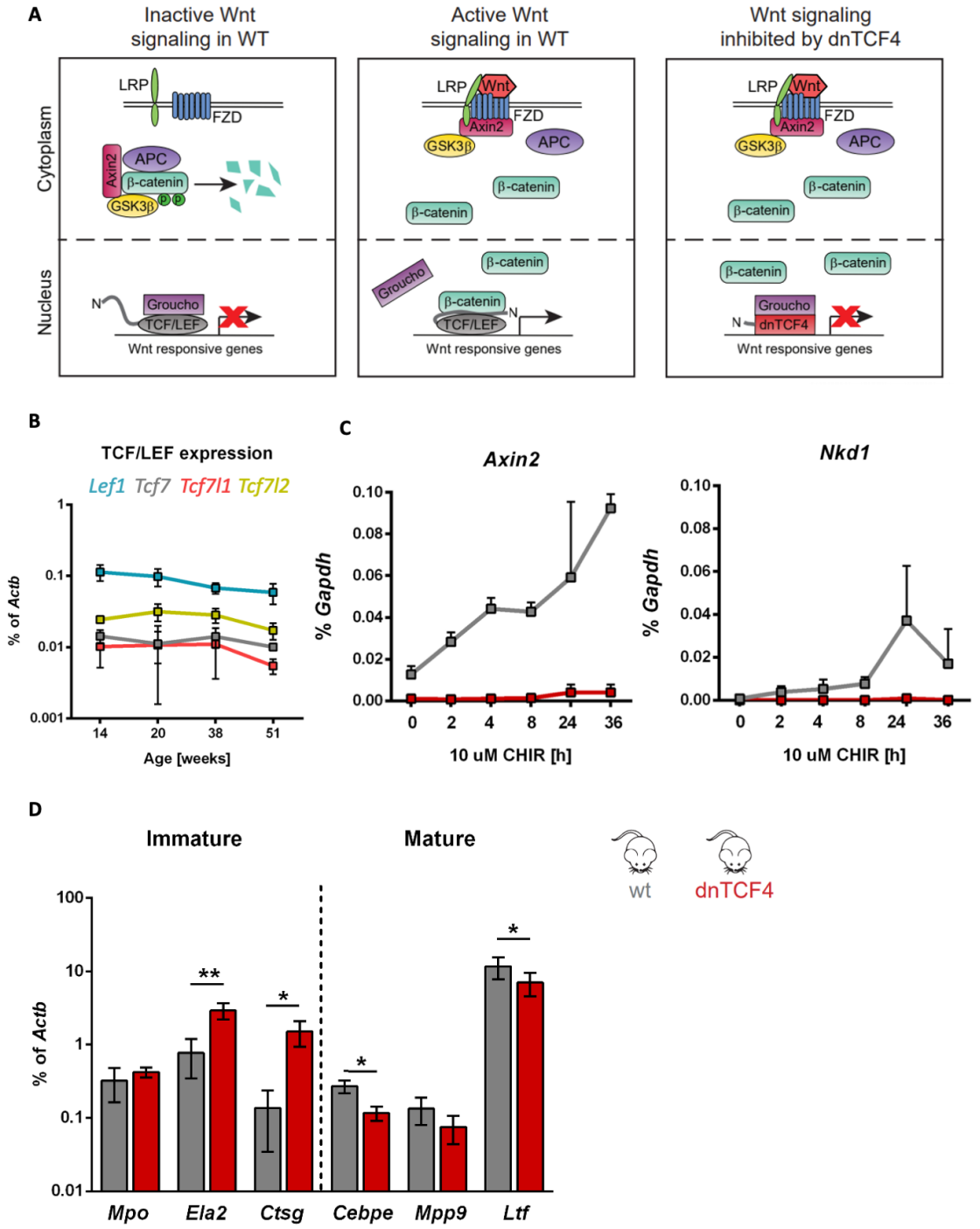
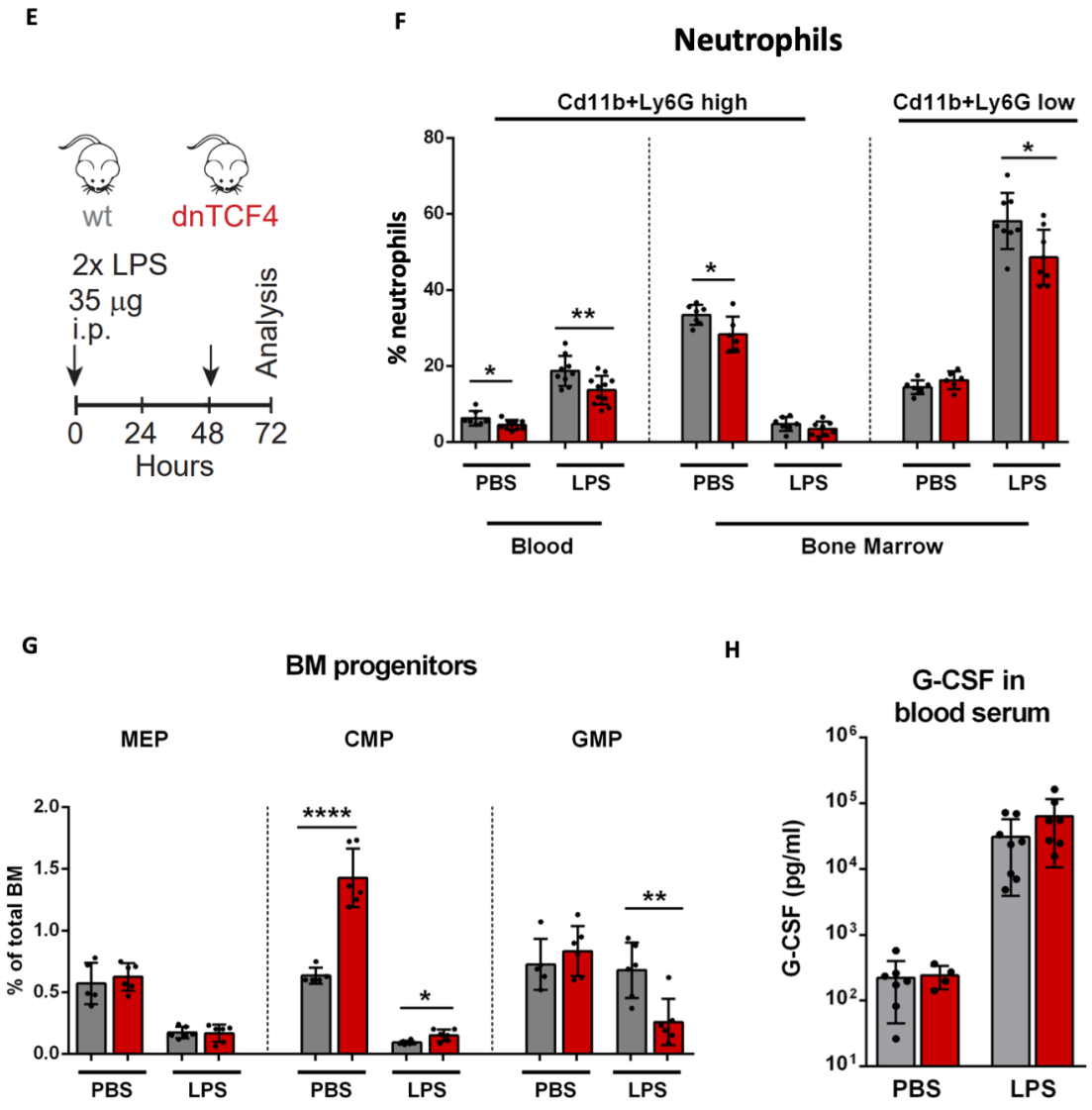


Figure 5. dnTCF4 introduction to the BM impairs EG response.
A. *Scheme of inactive β-catenin signaling (left), active β-catenin signaling (middle) and β-catenin transcription abrogated by the expression of dnTCF4 (right). Scheme done by Mgr. Petr Daněk, Ph.D.* ***B.*** *mRNA levels of 4 TCF/LEF factors in whole bone marrow of WT mice of different ages.* ***C.*** *mRNA levels of β-catenin target genes in WT or dnTCF4 mice following stimulation with activator of β-catenin signaling CHIR99021 for indicated timepoints.* ***D.*** *mRNA levels of granulocytic markers (left – immature, right – mature) in cells collected after a week of cultivation in semi-solid medium (colony culture assay).*



E. *Scheme of LPS treatment used to induce EG.* ***F.*** *Percentage of blood and bone marrow neutrophils of WT or dnTCF4 animals treated with PBS or LPS* ***G.*** *Percentage of BM progenitor populations (MEP, CMP, GMP) from WT and dnTCF4 animals treated with PBS or LPS.* ***H.*** *G-CSF levels (pg/ml) from blood serum collected from WT and dnTCF4 animals treated with PBS or LPS.*

In our first aim we validated that the dnTCF4 murine model is a valid model to be used in the investigation of β-catenin signaling in the hematopoietic compartment. The introduction of a dnTCF4 transgene in the hematopoietic compartment led to decreased granulocytic differentiation, which hindered successful response to LPS stimulation. This impaired EG response in the dnTCF4 animals was marked by significantly lower mobilization of granulocytes to the blood and decreased production of new granulocytes. Analysis of G-CSF levels in the serum of PBS/LPS treated mice showed no differences between WT and dnTCF4 suggesting that some other, cell intrinsic factor is blocking the successful EG response.

All the experiment presented in this figure have been carried out in cooperation with Mgr. Petr Daněk, Ph.D., who continued to study the dnTCF4 mouse model and the phenotype in the granulocytic compartment further. The results, including the data presented in this figure, were published recently [134].

Briefly, dnTCF4 animals accumulate immature cells as a result of a block in granulocytic differentiation. This block compromises EG response and the animals are more susceptible to *C. Albicans* and 5-FU treatments. Mechanistically, β-catenin/TCF/LEF transcriptional complex regulates the levels of G-CSF-R and therefore the lack of β-catenin mediated transcription in the dnTCF4 levels results in lowered G-CSF-R levels, causing decreased sensitivity to G-CSF signaling [134].

However, whether stimulation of EG is able to increase the active β-catenin levels in HSPCs directly is not known. In this thesis, we further aim to investigate whether induction of EG leads to increased β-catenin levels leading to activation of the canonical Wnt/β-catenin signaling pathway in HSPCs. We also aim to study the very early changes that emergency granulopoiesis induces at the level of HSPCs. For both of these aims, we decided to use WT animals, to avoid any genetic modification, that might influence the response.

8.2 Single LPS injection induces EG response

The EG model that was reported previously is used to mimic severe systemic infection and it includes two injections of LPS and analysis 72 hours after the first injection [34]. However, since we aim to investigate early changes that are induced immediately following LPS stimulation, we had to set up a different protocol using a single LPS injection that would allow us to study the changes at an early timepoint.

The EG program is initiated when there is an increased demand for granulocytes in the organism, which is usually caused by an infection. The goal of EG is to rapidly produce new granulocytes at high rates to ensure clearance of the pathogen from the system. However, a crucial step of EG is also the release of already mature granulocytes from the BM, which travel through blood to the site of infection. The egress of granulocytes from BM is very rapid and happens early after the stimulation and therefore can be employed as a parameter to determine the efficiency of EG. Two opposite but complementary parameters can be measured to assess the efficiency of EG: (1) number of granulocytes in BM cells (which is drastically reduced upon initiation of EG), and (2) number of granulocytes in blood (which is drastically increased upon initiation of EG). Another critical parameter that can be measured to assess EG is the expansion of immature granulocytes in BM.

In Figure 6 we treated WT mice with LPS and characterized the granulocytic response *in vivo* over time. We define mature granulocytes as Gr1 (Ly6G and Ly6C) Cd11b double positive population. This is the population that is not present in C/EBPα KO mice in steady state but can be observed when inducing EG suggesting that this is really the C/EBPβ induced population, and therefore our population of interest [72]. Immature granulocytes are also Cd11b+ but have intermediate expression of Gr1. The percentage as well as the composition of these populations changes with increasing time following LPS stimulation (Figure 6A). The levels of mature granulocytes in BM decreased significantly towards 4H following LPS stimulation, another significant decrease was detected at 24 H post stimulation (Figure 6B). The levels of immature granulocytes decrease slightly at 4H post stimulation and then starts increasing at 12H with the most significant increase at 24H (Figure 6C). The changes in the composition of the BM can be also detected by changes in absolute cell counts. The cell counts decrease rapidly as the cells start leaving the BM at early timepoints and increase slowly at later timepoints

as new cells are being produced (Figure 6D). The decrease in the BM granulocytes translates to the increase of granulocytes in blood (Figure 6E). However, the levels of granulocytes in blood showed variability at some timepoints which is most likely caused by the infiltration to the tissues.

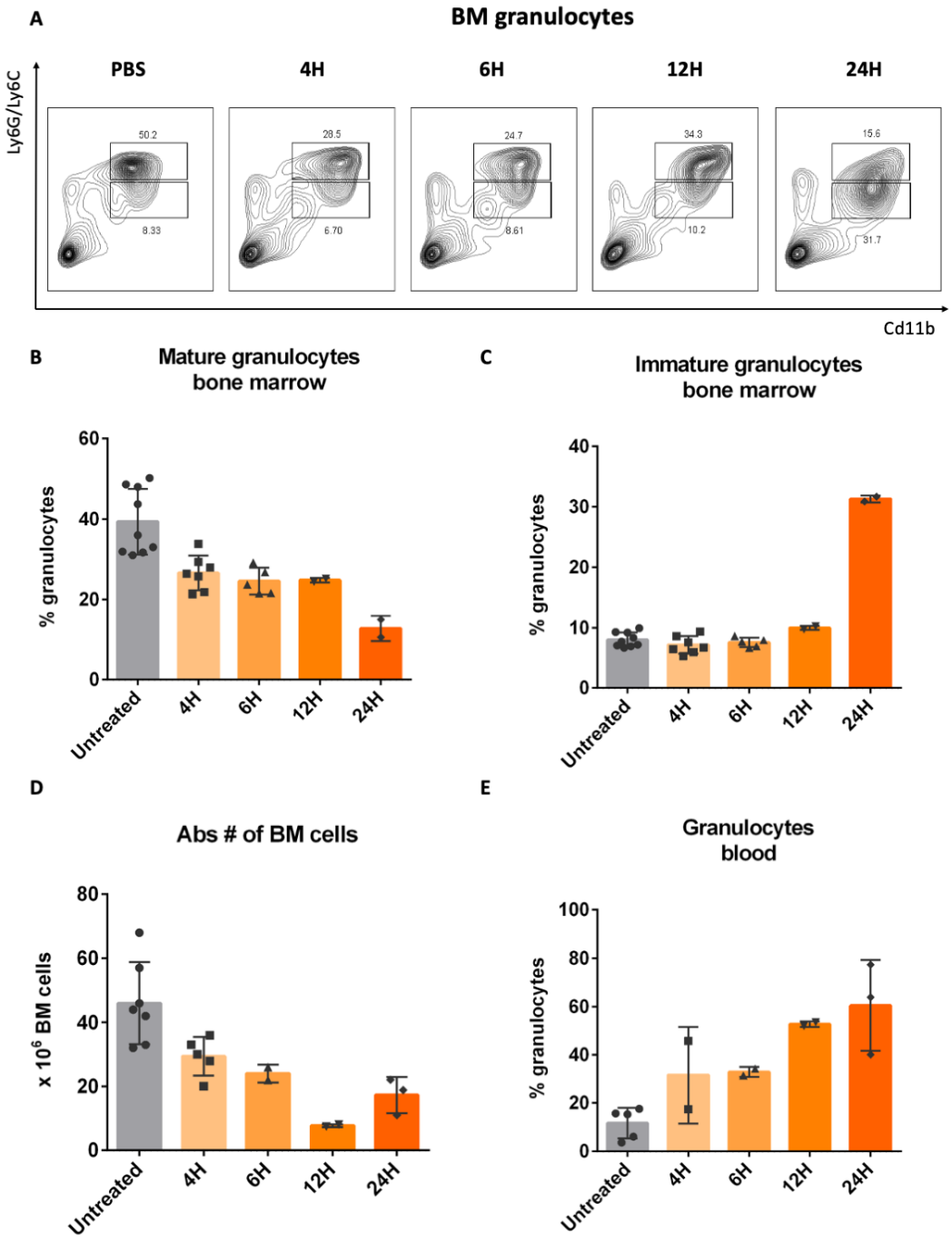


Figure 6. Granulocytic response following LPS treatment.
A. *Representative contour plots of BM granulocytes at different timepoints following LPS stimulation, populations gated: mature granulocytes (top) and immature granulocytes (bottom).* ***B.*** *Percentage of mature BM granulocytes at different timepoints.* ***C.*** *Percentage of immature BM granulocytes at different timepoints.* ***D.*** *Absolute BM cell counts at different timepoints.* ***E.*** *Percentage of granulocytes in blood at different timepoints.*

Altogether, the analysis of granulocytic response over time showed that a single LPS injection is sufficient to induce mobilization of mature granulocytes from the BM to blood at early timepoints (4H post stimulation), as well as the production of immature granulocytes at later timepoints (24H post stimulation).

8.3 Active β-catenin accumulates in HSPCs following LPS stimulation *in vivo*

dnTCF4 mice do not produce sufficient levels of granulocytes following LPS stimulation because of block at GMP level. To investigate whether β-catenin gets activated in HSPCs following LPS stimulation, we injected WT mice with LPS and analyzed them 24H later, at a timepoint when new granulocytes are already being produced.

First, we analyzed the levels of *Cebpb*, master regulator of EG, as a control for proper activation of the EG program. The levels increased significantly, suggesting that EG has been initiated (Figure 7A). To investigate the β-catenin activation we have employed FACS analysis using intracellular β-catenin staining. The use of an antibody that targets non-phosphorylated (=active) β-catenin allows us to determine the levels of active β-catenin levels in different populations. This analysis showed the accumulation of active β-catenin 24 H after *in vivo* administration of LPS (Figure 7B). The increase was detectable in both progenitors (Lin- c-Kit+ Sca1-) and stem cells (Lin- c-Kit+ Sca1+), as shown in Figures 7C and D, respectively. The increase was significant in both populations, although greater in the stem cell compartment, suggesting a crucial role for β-catenin signaling in these populations. We also tried to validate these finding by analyzing the expression of β-catenin target gene *Axin2,* however the mRNA levels did not increase in sorted c-Kit+ cells following *in vivo* stimulation (Figure 7E).
Together, these experiments indicate that upon induction of EG by LPS administration, the non-phosphorylated form of β-catenin gets accumulated, suggesting that the β-catenin transcriptional activity might increase (despite the fact that Axin2 was not upregulated).

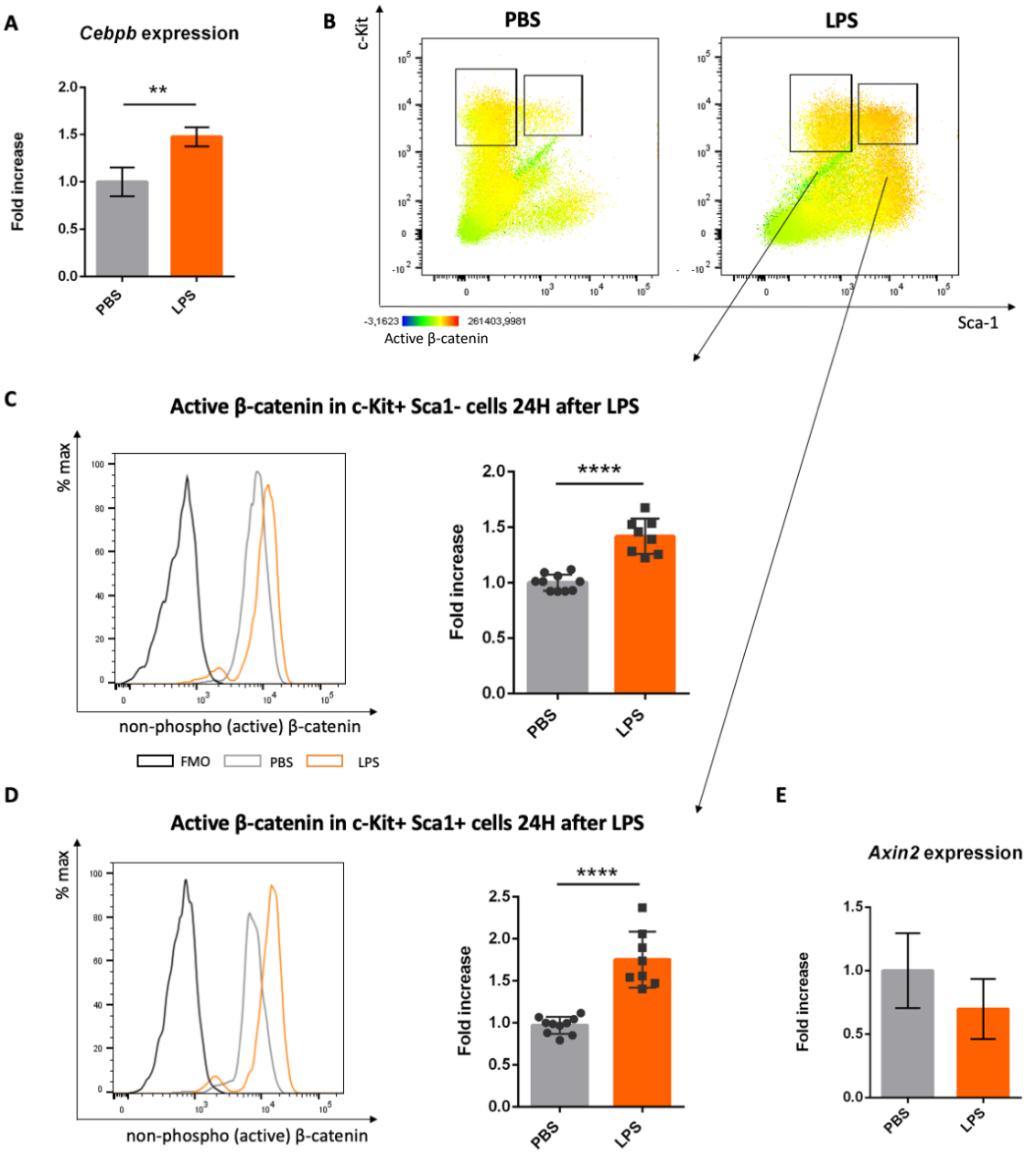


Figure 7. Active β-catenin gets accumulated in HPSC 24H after LPS treatment.
A. *mRNA levels of Cebpb in sorted c-Kit+ cells.* ***B.*** *Heatmaps of active β-catenin in lineage negative cells of PBS treated animals or 24H after LPS stimulation Gates represent c-Kit+Sca- cells (progenitors) and c-Kit+Sca1+ cells (stem cells).* ***C., D.*** *Histograms of active β-catenin levels and quantification of MFIs, shown as fold increase from PBS treated in c-Kit+Sca1- cells (**C.**) and c-Kit+Sca1+ cells (**D.**).* ***E.*** *mRNA levels of Axin2 in sorted c-Kit+ cells from PBS or LPS treated animals.*

8.4 Active β-catenin accumulates in c-Kit+ cells following LPS, but not Pam3CSK4, stimulation *in vitro*

Since we observed that LPS stimulation activates β-catenin *in vivo*, we next determined whether there is a direct activation, or an indirect activation mediated by the niche and potentially the overall inflammatory state that is induced in the animals. To assess that we sorted c-Kit+ cells from untreated WT animals, stimulated them *in vitro* with LPS and checked for β-catenin activation by intracellular β-catenin staining, as well as by upregulation of the target gene *Axin2* at different timepoints.

We observed that β-catenin was upregulated already at 2H following LPS stimulation, with highest levels at 4H. This increase was sustained at 6H and decreased towards 24H. We were able to show β-catenin activation only by intracellular staining (Figure 8A, B), as there was again no increase in *Axin2* levels (Figure 8C).

Interestingly, by doing a similar time course experiment using another TLR ligand – triacylated lipopeptide Pam3CSK4, ligand of TLR2/1, we were able to show that the activation is specific for LPS, but not Pam3CSK4. The levels of active β-catenin did not increase at any analyzed timepoint following Pam3CSK4 stimulation *in vitro* (Figure 8D).

Altogether, the experiments presented so far compiled our results related to aim 1. To summarize this first part, we observed that dnTCF4 expression abrogates β-catenin mediated transcription in the hematopoietic compartment and this causes a block in granulocytic production and impairs EG response at the level of GMPs. Further, we showed that β-catenin gets activated in HSPCs during EG both *in vivo* and *in vitro.* This activation is detectable only by intracellular β-catenin staining and not by upregulation of β-catenin target genes. Finally, we showed that β-catenin activation is at least partially independent of the niche and is LPS/TLR4 specific.

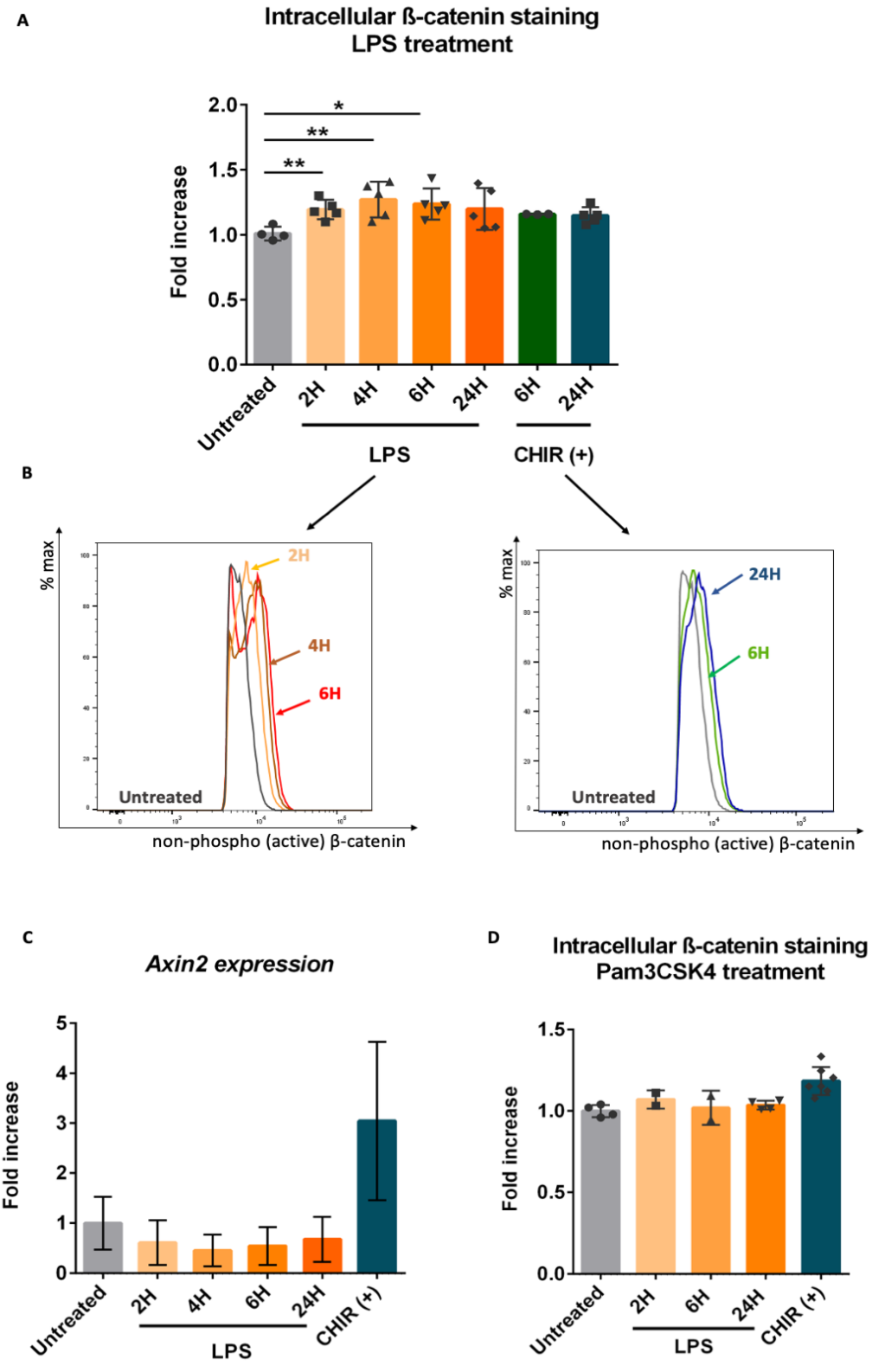


Figure 8. β-catenin activation in c-Kit + cells peaks at 4h following in vitro LPS stimulation. ***A., B.*** *MFI quantification (A.) and representative histogram (B.) of β-catenin activation at different timepoints after LPS stimulation. CHIR – positive control* ***C.*** *mRNA levels of Axin2 at different timepoints after stimulation.* ***D.*** *MFI quantifications of active β-catenin levels at different timepoints of Pam3CSK4 stimulation.*

8.5 Emergency granulopoiesis is induced in HSPCs 4 hours after LPS stimulation

Our second aim was to identify early transcriptional changes that occur in HSPC after inducing EG using scRNA-seq. First, we had to select the right timepoint after induction of the EG program. Based on the granulocytic response, we decided for T = 4h after 1 dose of LPS administration, in Figure 9 we further validated the timepoint.

In Figure 8 we showed that the dynamic of β-catenin activation is quite fast and peaks at 4H *in vitro*, therefore we assessed whether we could detect β-catenin accumulation at 4H *in vivo* as well. This would allow us to investigate β-catenin induced changes in the scRNA-seq experiment. Indeed, we detected accumulation of active β-catenin in both progenitors and stem cells (Figure 9A), but as expected the levels were lower than the levels detected at 24 hours (Figures 7C-D).

Lastly, since we aimed to analyze gene expression 4 hours after LPS stimulation, we determined whether transcriptional changes were indeed initiated. Therefore, we checked the mRNA levels of three important regulators of EG: *Cebpb, Bcl3* and *Il6* using quantitative RT-PCR (Figure 9B). *Cebpb* and *Bcl3* were significantly upregulated and *Il6* showed similar trend, however, didn't reach significance because of big standard deviation. The mRNA levels of these EG-related genes were far greater 4 hours after LPS than the levels at 12H (a timepoint when new immature granulocytes begin to arise in the BM). This suggest not only that EG has been initiated, but that 4H is a very early timepoint of EG, based on the mRNA levels of EG regulators.

Thus, for scRNA-seq mice were injected with PBS or LPS, sacrificed 4 hours later. MPPs (Lin- c-Kit+ Sca1+ CD48+ CD150-) and HSCs (Lin- c-Kit+ Sca1+ CD48- CD150+) were sorted and viability was assessed to ensure at least 96% viable cells. Further, libraries were prepared with the target of 4000 cells and sequenced in 2 runs of NextSeq 500 sequencer using NextSeq 500/550 high output kit v2.5 Of note, we also assed the granulocytic response of the animals used for the experiment, to ensure that EG was initiated (Figure 9C). We have observed higher percentage of granulocytes in the blood than presented in Figure 6, however this was not surprising, since the percentage of granulocytes in the blood was always quite variable and the other criteria showed similar response as presented in Figure 6.

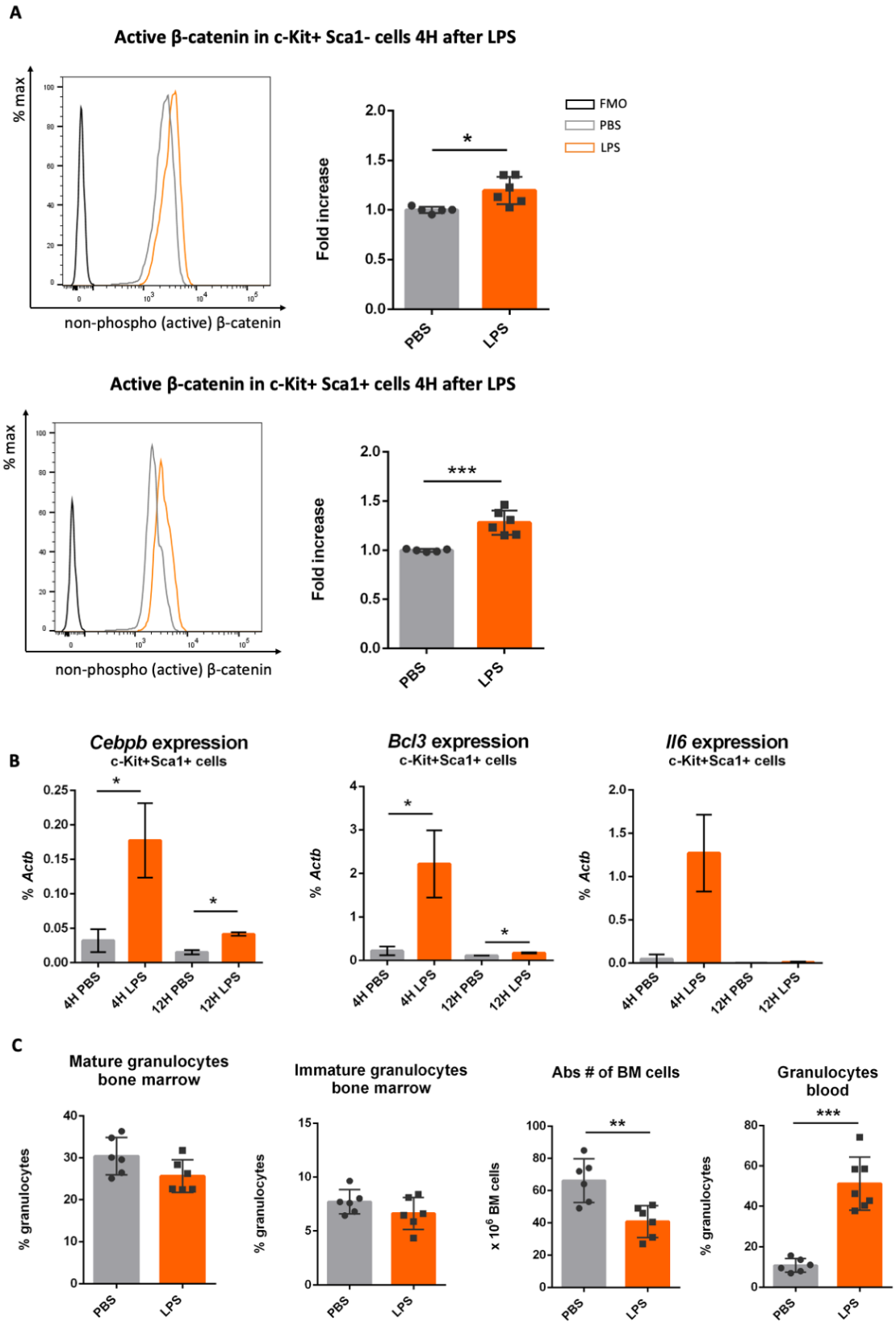


Figure 9. EG is initiated already at 4 hours following LPS stimulation.
A. *Histograms and their quantifications showing levels of active β-catenin 4 hours after LPS stimulation in c-Kit+Sca-1- cells (top) or c-Kit+Sca-1+ cells (bottom).* ***B***. *mRNA levels of Cebpb, Bcl3 and Il6 4 or 12 H following in vivo LPS treatment.* ***C***. *Granulocytic response of mice used for scRNA-seq (same criteria as in Figure 6).*

8.6 scRNA-seq of MPPs revealed distinct progenitor sub-populations responsible for the early activation of EG

The first population we analyzed using scRNA-seq was MPPs. UMAP projection of cell clustering is shown in Figure 10A. We expected the MPP sample to be a mixture of different progenitor populations and indeed, 10 clusters (sub-populations) were identified in the PBS and LPS treated samples. Of note, clusters are formed by cells exhibiting a similar gene expression profile. Interestingly, the distribution and density of the clusters did not differ greatly between PBS (left) and LPS (right). The major differences are highlighted by an arrow. Clusters numbers 2 (green) and 4 (purple) seem to be PBS specific and disappear following LPS treatment. Reversely, cluster 10 (orange) is only present in LPS treated sample. Sub-populations 7 (gray) and 9 (light blue) are composed of fewer cells in PBS treated sample and get greatly enlarged following LPS stimulation, suggesting that these clusters might also be important for the emergency response.

In order to define the distinct sub-populations, we identified using scRNA-seq, we performed Gene Set Enrichment Analysis (GSEA) for each of the identified clusters. GSEA is a tool used to compare gene expression in a certain populations with specifically curated gene sets that define individual cell types, pathways, signatures etc., thus allowing for easy interpretation of gene expression data [135]. First, we performed the GSEA with gene sets that define individual MPP populations, published by Pietras *et al*. [26] and then we validated these findings using Gene Ontology (GO).

Inside the MPP population distinct sub-populations have been identified [26]. MPP2 gives rise predominantly to megakaryocytic and erythroid lineage, MPP3 give rises to granulocytes and monocytes, and MPP4 represent lymphoid progenitors. Interestingly, we were able to assign 7/10 clusters to one of the MPP populations (Figure 10B, top part of the heatmap).

To further confirm these findings, we performed GSEA with the Gene Ontology geneset to identify Cellular components (GOCC), Molecular function (GOMF) and Biological processes (GOBP) that are significantly (FDR <0,25) up and downregulated in these clusters (Figure 10B, bottom part of the heatmap). Surprisingly, two out of the three clusters that did not show signature of any MPP population, showed strong megakaryocytic signature based on the GO, while still keeping a signature of HSC

homeostasis. The last unassigned cluster had strongly downregulated all the genesets employed, so the identity of this sub-population (cluster 1) remains unknown.

Further, a significant part of the MPP sample showed lymphoid potential (clusters 2, 4, 6). Interestingly these were the clusters that were present in the PBS sample and disappeared following LPS treatment. Reversely, the LPS-specific clusters 9 and 10 showed a myeloid signature and high inflammatory signature, suggesting that these two populations are the ones that are orchestrating the emergency response. Remarkably, sub-populations characterized by a megakaryocytic and erythroid profile (clusters 3 and 8) and correlating to the MPP2 signature did not change much between the PBS and LPS condition, suggesting their low contribution to the early activation of EG program.

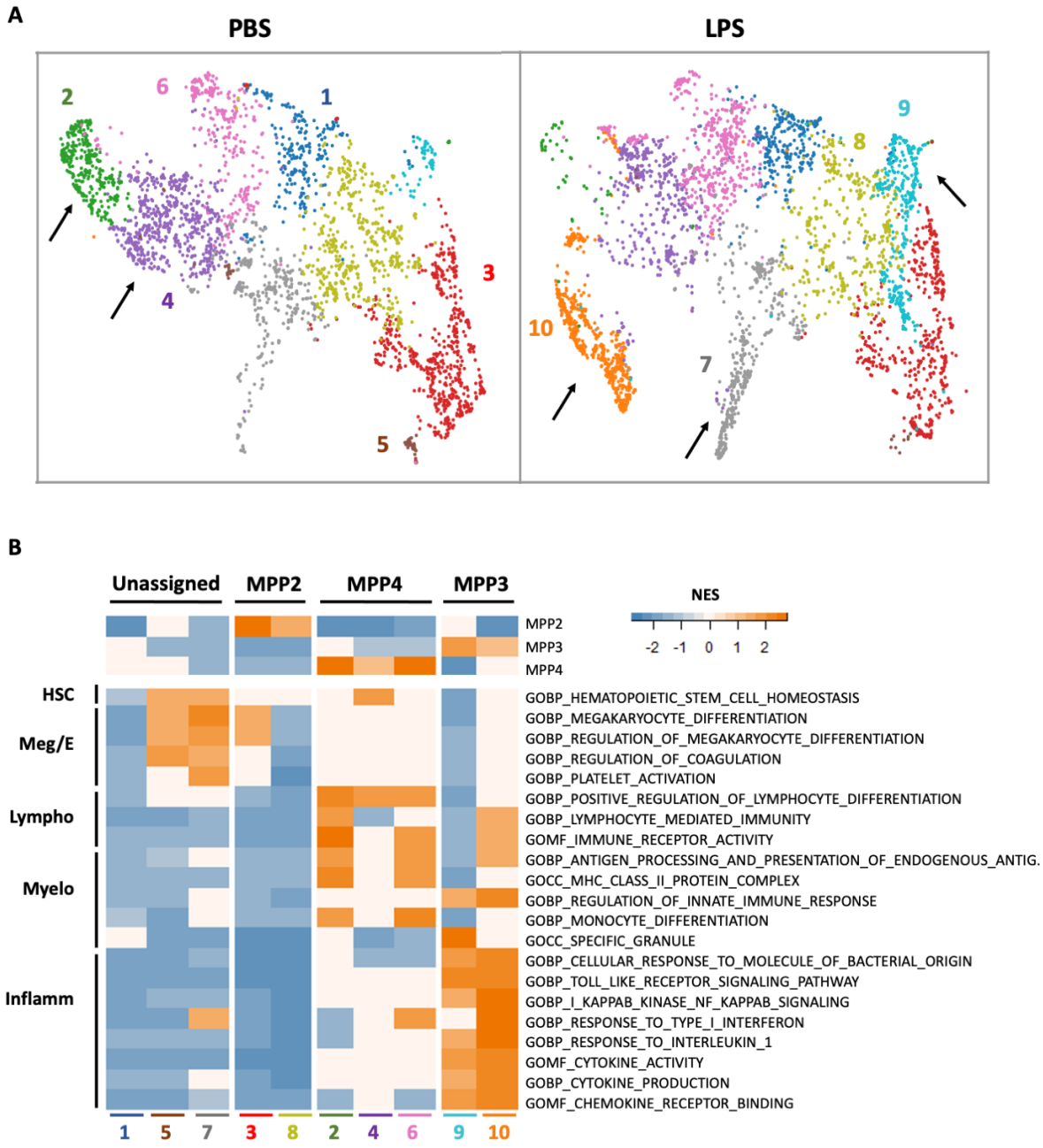


Figure 10. scRNA-seq of the MPP compartment upon PBS and LPS administration.
A. *UMAP plots of MPPs sorted from mice treated with PBS (left) and LPS (right). Different clusters are shown in different colors and major changes between PBS and LPS sample are indicated by an arrow.* ***B****. Heatmaps of gene sets significantly enriched in individual clusters based on NES (normalized enrichment score). 0 equals non-significant (FDR > 0,25). Meg/E: Megakaryocyte/Erythroid, Lympho: Lymphoid, Myelo: Myeloid, Inflamm: Inflammatory.*

8.7 scRNA-seq of HSCs revealed expansion of myeloid bias sub-populations upon LPS administration

The scRNA-seq analysis of the HSC compartment also revealed multiple clusters, but unlike the MPP compartment, the cluster composition changed drastically following LPS stimulation. Strikingly, most of the clusters present in the PBS sample (1, 3, 4, 6) were not present in the LPS sample, and instead 3 clusters (8, 9, 10) appeared following the stimulation (Figure 11A). Surprisingly, we were also able to identify signatures of MPP subsets in the HSC compartment (Figure 11B, top part of heatmap). However, as expected, the individual signatures were not as strongly upregulated in comparison with the MPP sample. Nevertheless, this suggests that some level of lineage bias is already present at the level of stem cells.

Clusters 2, 5 and 7 showed an MPP2 signature (megakaryocytic and erythroid lineage), and similarly to the situation in the MPP analysis in PBS and LPS conditions, these were the only three clusters that remained largely unchanged following LPS stimulation. Again, this suggests that sub-populations with a megakaryocytic and erythroid potential are preserved and remain largely unaffected in early stages of EG.

Clusters 3 and 6 showed an MPP4 (lymphoid) signature and vanished upon LPS administration. However, GSEA defined them as sub-populations that were enriched for genes related to HSC homeostasis (Figure 11B, bottom part of heatmap). This could mean that these stem cells are shifting from a lymphoid bias to a more immature phenotype, and thus preserve the true HSC pool under stress conditions. Or alternatively that they are primed to give rise to lymphoid cells but can still develop into different lineages. This could be further supported by the 3 clusters that appear following LPS stimulation. All of them showed MPP3 (myeloid) signature, while 2 of them also retained the MPP4 signature. We hypothesize that these cells origin from the cells from clusters 3 and 6. This would suggest that during stress conditions the lymphoid progenitors get reprogrammed and start producing myeloid cells that are necessary to fight the infection.

Further, in the HSC compartment we also identified two sub-populations (cluster 1 and 4) which identity or nature remain unknown. Of note, their abundance and representation were reduced upon LPS administration, suggesting that they might be rewired into another sub-population type.

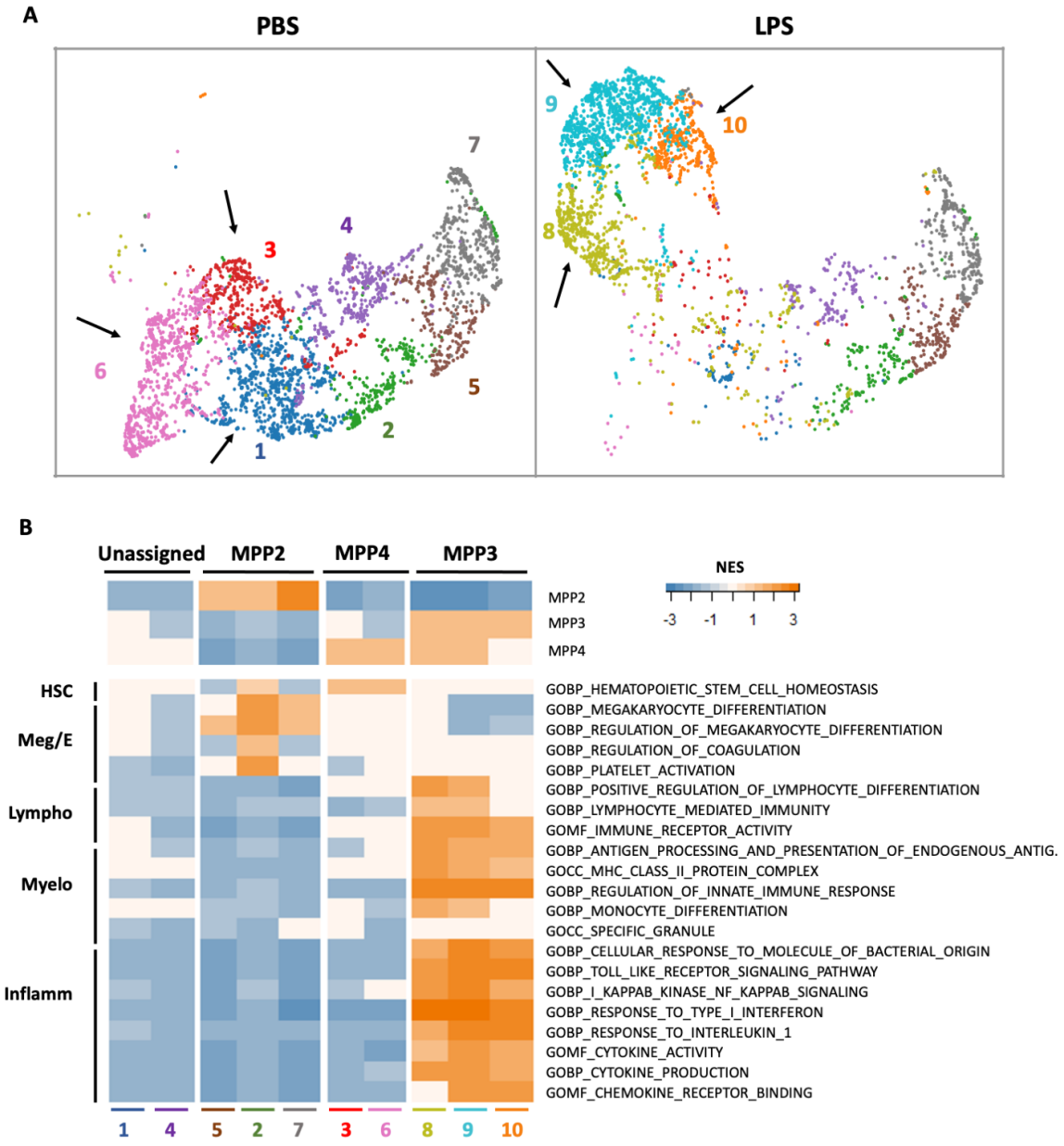


Figure 11. scRNA-seq analysis of the HSC compartment upon PBS and LPS administration. ***A****. UMAP plots of HSCs sorted from mice treated with PBS (left) and LPS (right). Different clusters are shown in different colors and major changes between PBS and LPS sample are indicated by an arrow.* ***B****. Heatmaps of gene sets significantly enriched in individual clusters based of NES (normalized enrichment score). 0 equals non-significant (FDR > 0,25). Meg/E: Megakaryocyte/Erythroid, Lympho: Lymphoid, Myelo: Myeloid, Inflamm: Inflammatory.*

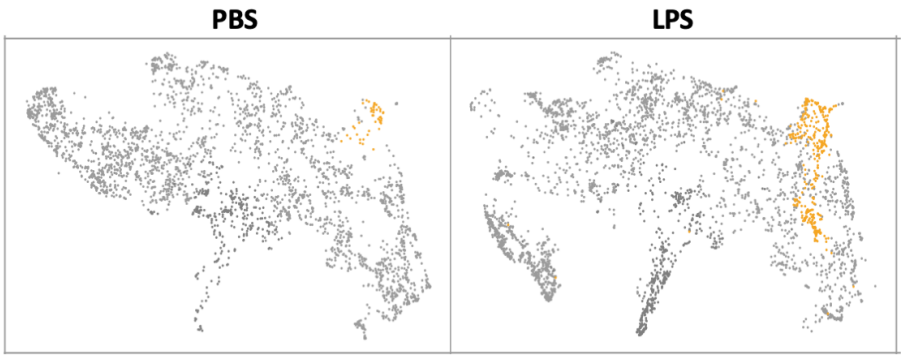
8.8 MPP cluster 9 is a granulocytic cluster that is marked by enrichment of β-catenin target genes

In the MPP sample, we identified a sub-population (cluster 9) that was composed of very few cells in the PBS treated sample and got greatly enlarged following LPS stimulation (Figure 12A, shown in orange). Further, GO analysis showed enrichment in GO set "specific granules" (Figure 10B). We performed further GSEA analysis and indeed, we identified multiple granulocyte specific, as well as multiple inflammatory GO sets amongst the top 25 enriched GO terms (Figure 12B, shown in orange). Therefore, we hypothesize that cluster 9 might be a sub-population of MPPs that is the first one to drive the enhanced production of granulocytes following LPS stimulation.

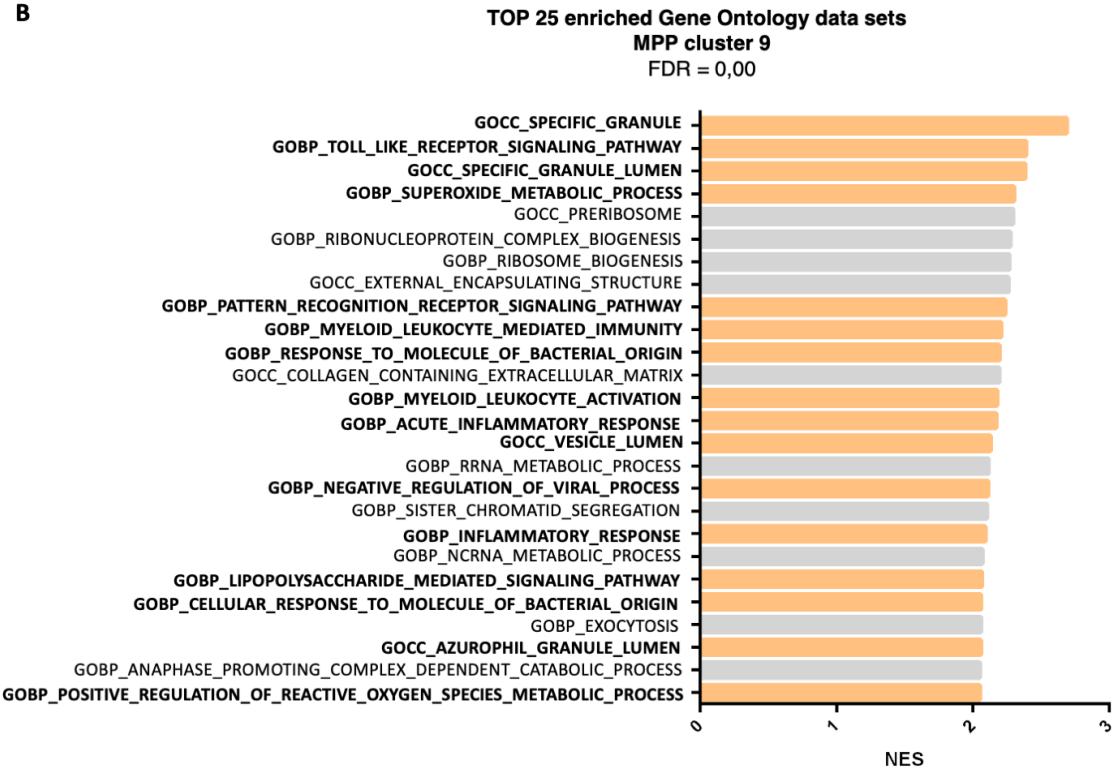
Next we sought to investigate the pathways that are upregulated in this cluster, using the Pathway interaction database (PID) [136]. We identified 5 pathways that were enriched in cluster 9 (Figure 12C). Amongst them we found Aurora kinase A pathway. Interestingly, Aurora kinase A has been identified as a β-catenin target gene in multiple myeloma disease [137]. However, we did not find enrichment in the Wnt signaling pathway. Since our results in aim 1 showed that at 4 hours upon LPS stimulation β-catenin is already accumulating at protein level, it is possible that β-catenin is already regulating gene expression, so we decided to check β-catenin target genes. There are two datasets including β-catenin target genes in the Molecular Signature Database (MSigDB). The first one (KENNY_CTNN1B_TARGET_GENES_UP) represents genes that are activated in mammary epithelium by β-catenin [138]. Using this gene set, we found significant enrichment of β-catenin target genes in the granulocytic cluster (Figures 12D, E). The second gene set (FEVR_CTNN1B_TARGETS_DN) represents genes that are downregulated following β-catenin deletion in intestinal crypt cells [139]. This gene set confirmed the upregulation in the granulocytic cluster and identified β-catenin target genes in two additional clusters (cluster 3 and 8) (Figures 12F, G). Interestingly, both of these were clusters that showed MPP2 signature and on the UMAP plot surround the granulocytic cluster.

Together, based on these observations we hypothesize that β-catenin participates in promoting EG in a sub-population of MPPs (cluster 9) which has MPP3 identity, is significantly expanded upon LPS exposure, and is characterized by expression of myeloid and inflammatory genes.

A


PBS
LPS

B


TOP 25 enriched Gene Ontology data sets
MPP cluster 9
FDR = 0,00
GOCC_SPECIFIC_GRANULE
GOBP_TOLL_LIKE_RECEPTOR_SIGNALING_PATHWAY
GOCC_SPECIFIC_GRANULE_LUMEN
GOBP_SUPEROXIDE_METABOLIC_PROCESS
GOCC_PRERIBOSOME
GOBP_RIBONUCLEOPROTEIN_COMPLEX_BIOGENESIS
GOBP_RIBOSOME_BIOGENESIS
GOCC_EXTERNAL_ENCAPSULATING_STRUCTURE
GOBP_PATTERN_RECOGNITION_RECEPTOR_SIGNALING_PATHWAY
GOBP_MYELOID_LEUKOCYTE_MEDIATED_IMMUNITY
GOBP_RESPONSE_TO_MOLECULE_OF_BACTERIAL_ORIGIN
GOCC_COLLAGEN_CONTAINING_EXTRACELLULAR_MATRIX
GOBP_MYELOID_LEUKOCYTE_ACTIVATION
GOBP_ACUTE_INFLAMMATORY_RESPONSE
GOCC_VESICLE_LUMEN
GOBP_RRNA_METABOLIC_PROCESS
GOBP_NEGATIVE_REGULATION_OF_VIRAL_PROCESS
GOBP_SISTER_CHROMATID_SEGREGATION
GOBP_INFLAMMATORY_RESPONSE
GOBP_NCRNA_METABOLIC_PROCESS
GOBP_LIPOPOLYSACCHARIDE_MEDIATED_SIGNALING_PATHWAY
GOBP_CELLULAR_RESPONSE_TO_MOLECULE_OF_BACTERIAL_ORIGIN
GOBP_EXOCYTOSIS
GOCC_AZUROPHIL_GRANULE_LUMEN
GOBP_ANAPHASE_PROMOTING_COMPLEX_DEPENDENT_CATABOLIC_PROCESS
GOBP_POSITIVE_REGULATION_OF_REACTIVE_OXYGEN_SPECIES_METABOLIC_PROCESS
0
1
2
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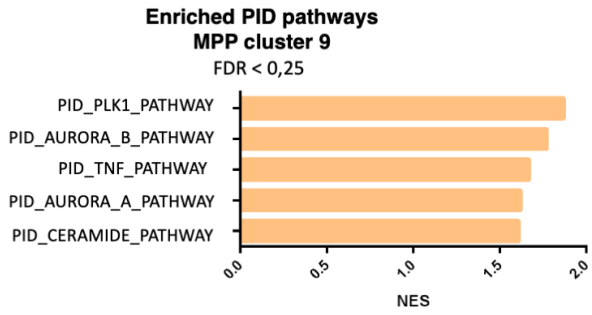
Enriched PID pathways
MPP cluster 9
FDR < 0,25
PID_PLK1_PATHWAY
PID_AURORA_B_PATHWAY
PID_TNF_PATHWAY
PID_AURORA_A_PATHWAY
PID_CERAMIDE_PATHWAY
0.0
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1.5
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NES

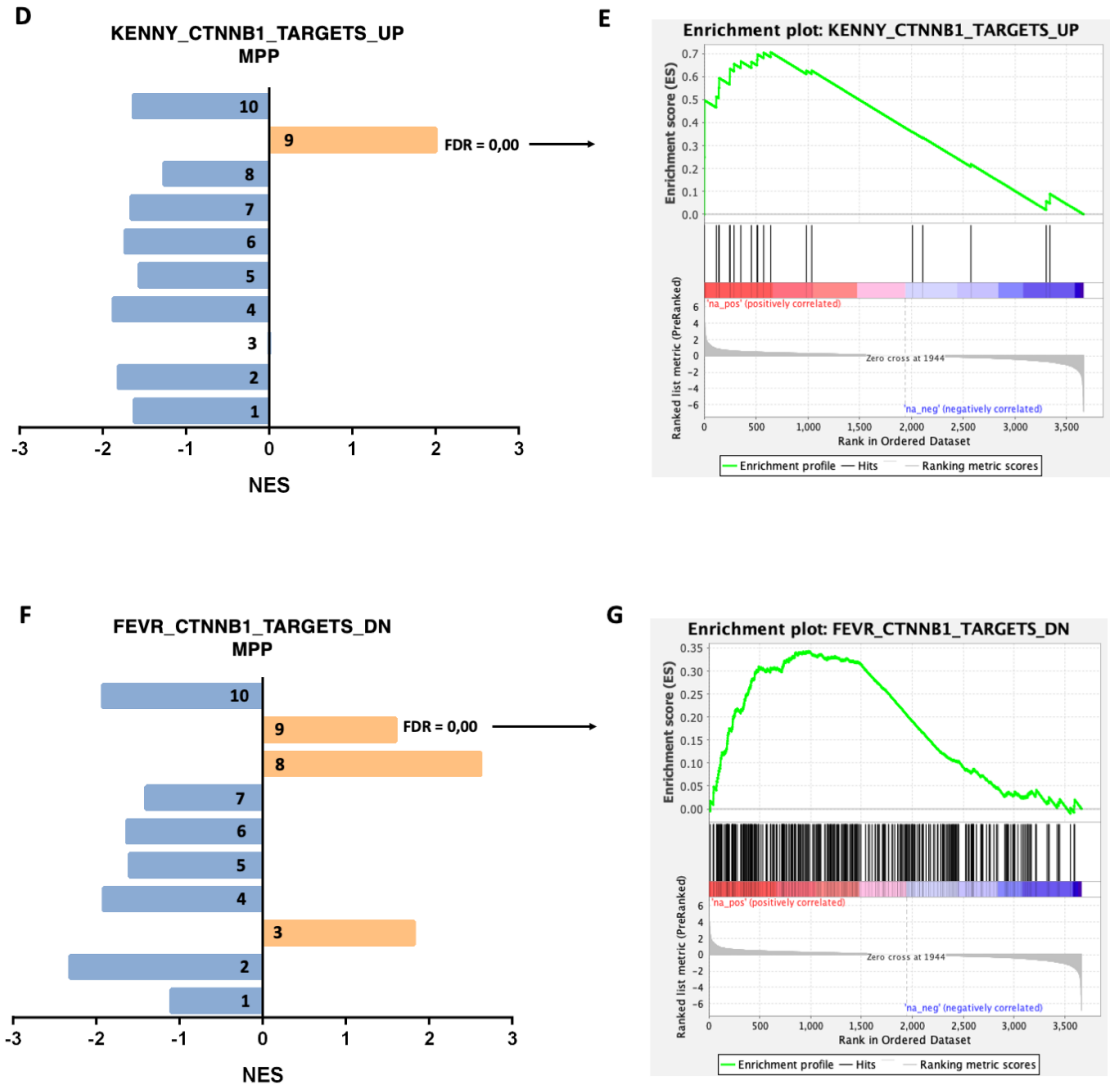

Figure 12. β-catenin target genes are enriched in a sub-population of MMPs characterized by exhibiting granulocytic identity.

A. *UMAP plots of MPP sample with the granulocytic cluster 9 in orange.* ***B.*** *TOP 25 GO terms significantly upregulated (FDR = 0,00) in cluster 9 of the MPP sample. Granulocytic and inflammation related GO terms are shown in orange* ***C.*** *Enriched PID terms (FDR < 0,25) in cluster 9 of the MPP sample.* ***D.*** *NES score of β-catenin target genes from gene set KENNY_CTNNB1_TARGETS_UP in each cluster of the MPP sample (blue downregulated, orange upregulated, 0 = non-significant).* ***E.*** *GSEA plot for cluster 9 (KENNY_CTNNB1_TARGETS_UP).* ***F.*** *NES score of β-catenin target genes from gene set FEVR_CTNNB1_TARGETS_DN in each cluster of the MPP sample.* ***G.*** *GSEA plot for cluster 9 (FEVR_CTNNB1_TARGETS_DN).*

8.9 A possible activator of β-catenin signaling *Wnt10b* is upregulated in HSCs during EG

One of the main questions that came up after we published our findings about β-catenin was what activates β-catenin signaling during EG. Thus, we screened out data for β-catenin related genes, Wnt ligands, and Frizzled receptors. Strikingly, we found a significant upregulation of *Wnt10b* in clusters 9 and 10, which appear in the HSC sample upon LPS treatment and are characterized by a myeloid and inflammatory signature (Figure 13A). Similarly, Wnt10b was upregulated in certain MPP sub-populations, also characterized by a myeloid identity, upon LPS stimulation. Interestingly, the expression profile of *Wnt10b* is very similar to that of *Il6* (Figure 13B). Given that IL-6 is an important inducer of EG that stimulates EG in a paracrine manner might indicate a similar role for Wnt10b.

To verify the upregulation of *Wnt10b* following LPS stimulation, we stimulated c-Kit+ cells *in vitro* with LPS for indicated timepoints. Interestingly, the levels of *Wnt10b* increased significantly at 2 and 4 hours following the stimulation (Figure 13C). This trend is almost identical to the upregulation of *Cebpb* that was observed in the same settings (Figure 13D) and coincides with the activation of β-catenin starting at 2H following LPS stimulation (Figure 7A). Lastly, we also verified whether Wnt10b stimulation is able to induce β-catenin activation. We stimulated c-Kit+ cells *in vitro* with recombinant Wnt10b protein and assessed the active β-catenin levels at different timepoints (Figure 13E). As expected, Wnt10b induced β-catenin activation at all timepoints analyzed with the biggest increase at the latest timepoint 24H. Together, these data indicate that Wnt10b might be an important regulator of the EG response and an upstream activator of β-catenin signaling in the HSPCs.

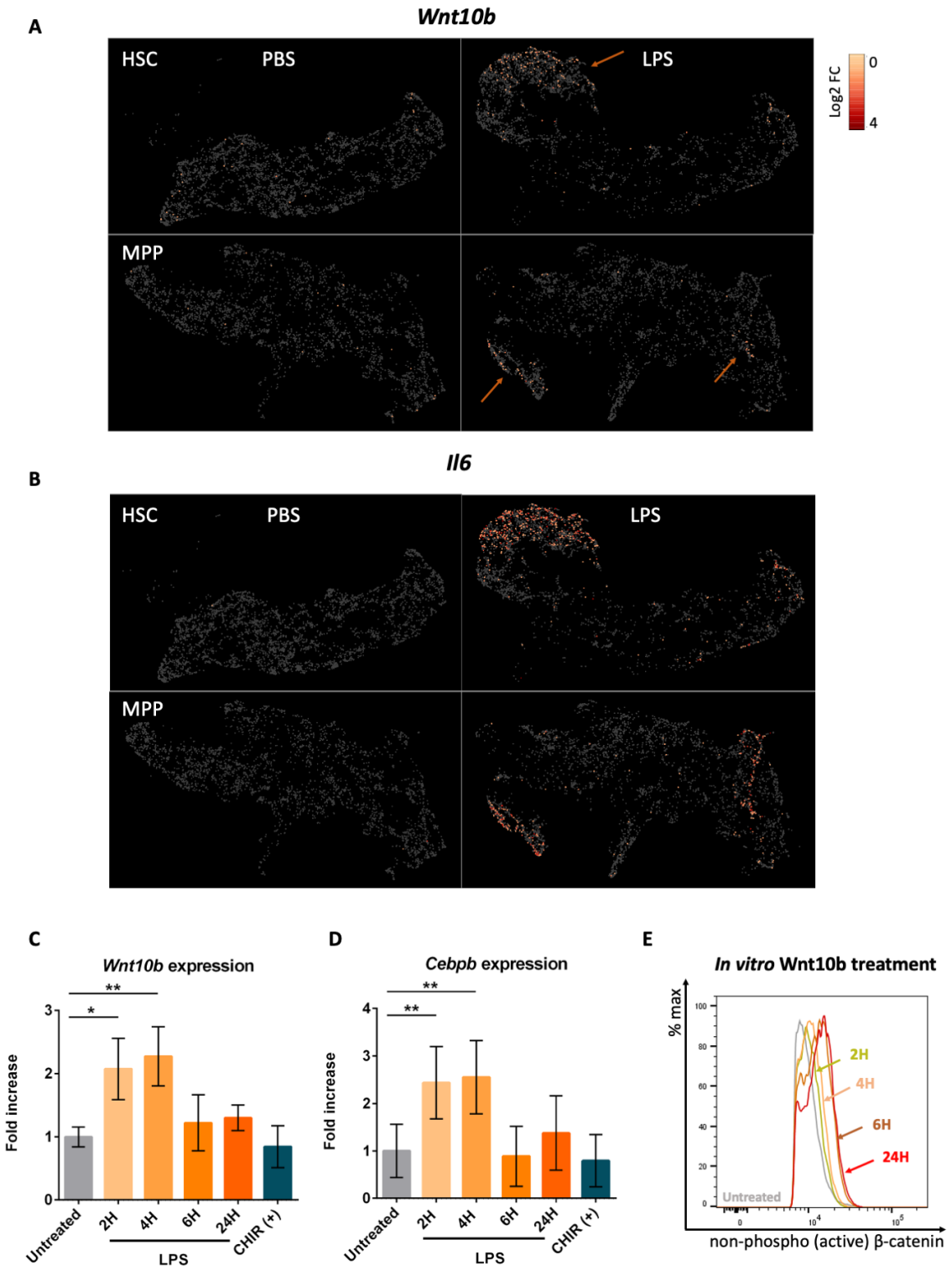

Figure 13. Wnt10b is upregulated in certain sub-populations in HSCs and MPPs during EG. ***A., B.*** *UMAP plots of Wnt10b (**A**) and Il6 (**B**) expression in HSCs (top) and MPPs (bottom) before and after LPS treatment. Heatmap shows Log2 fold change.* ***C., D.*** *mRNA levels of Wnt10b (**C**) and Cebpb (**D**) at different timepoints of LPS stimulation of c-Kit+ cells in vitro.* ***E.*** *Histogram of active β-catenin at different timepoints following in vitro Wnt10b stimulation of c-Kit+ cells.*

9 Discussion

The overall goal of this thesis was to get novel mechanistic insights into the process of emergency granulopoiesis and investigate the early changes that happen in HSPCs that may play a role in regulating the process that is crucial to protect our organism from bacterial and fungal pathogens. We focused specifically on the β-catenin signaling pathway, because it is one of the more controversial pathways studied in hematopoiesis and our preliminary results suggested that it might be important for SSG and EG.

The first aim of the thesis was to validate our newly generated dnTCF4 mouse model and determine the impact of β-catenin signaling deficiency on granulopoiesis. In this model, the β-catenin signaling is abrogated by the expression of a dnTCF4 transgene which lacks the binding transactivation domain for β-catenin, and consequently prevents β-catenin mediated transcription. Using the dnTCF4 mouse model, we identified decrease in maturity of the dnTCF4 cells compared to WT cells. The dnTCF4 BM cells have decreased granulocytic differentiation and retain a rather immature phenotype. Indeed, the dnTCF4 mice had decreased levels of granulocytes both in blood and BM and, when challenged with LPS, failed to induce a full granulocytic response necessary for successful EG. The block in granulocytic production was identified at the level of GMPs – the dnTCF4 mice had significantly decreased percentage of GMPs following LPS treatment, therefore the myeloid progenitor expansion, a crucial step for EG [4], was impaired by the β-catenin signaling deficiency. Myeloid progenitor expansion is induced by G-CSF [140], but no difference in G-CSF levels was found between WT and dnTCF4 animals in the blood serum during PBS or LPS treatment, suggesting cell intrinsic defects in the dnTCF4 cells. However, we cannot exclude that other soluble factors required for proper EG are reduced in the dnTCF4 mice contributing to the observed phenotype. Further experiments should explore differential expression of circulating factors, such as chemokines, cytokines, and Wnt ligands during EG in dnTCF4 mice in comparison to WT mice.

Further work with the dnTCF4 mouse model was carried out by Mgr. Petr Daněk, Ph.D. Indeed, β-catenin signaling is necessary for proper EG response, as the dnTCF4 animals showed worsened survival when challenged with *C. Albicans* and worsened recovery and survival following 5-FU treatment [134]. This was in agreement with the observations by Lento *et al*. who observed decreased BM cellularity marked by decreased levels of

granulocytes following single 5-FU treatment and decreased survival of β-catenin null mice following repeated 5-FU treatment [119].

Research using the dnTCF4 mouse model showed a block at the level of myeloid progenitors. However, we hypothesized that changes that drive EG response should be present already at the level of stem cells and multipotent progenitors early after induction of the EG response. Here, we established a model of EG based on one injection of LPS which allows us to investigate changes that occur in the HSPC compartment as early as 4 hours after LPS treatment. Using this model, we first investigated whether β-catenin gets activated in HSPCs following LPS treatment. To do that, we employed two methods – first we tried to assess β-catenin signaling activation by checking the mRNA levels of β-catenin target gene *Axin2*, unfortunately we were not able to show the activation of β-catenin signaling using this method *in vitro* or *in vivo*. This is probably because β-catenin signaling is a complex pathway with many regulatory loops and the target genes are not well described (especially in the hematopoietic compartment) [141]. So, using *Axin2* or target genes in general might not be a reliable way of assessing whether the pathway has been activated or not. Because of that we decided on an alternative approach. We employed intracellular β-catenin staining using an antibody that targets only non-phophorylated (active) β-catenin. This non-phosphorylated form accumulates in the cytoplasm first, and then translocates to the nucleus where it can bind the TCF/LEF TF and mediate transactivation of β-catenin target genes. Using this method, we were able to show that β-catenin gets accumulated in both progenitor and stem cell compartments. This was in agreement with previous studies showing accumulation of active β-catenin in HSPCs in different stress conditions, namely 5-FU treatment, irradiation or Cy/G treatment [58], [119].

The populations we use to analyze β-catenin activation are based on lineage, c-Kit, and Sca-1 markers. The lineage- double positive population is referred to as LSK and is enriched for stem cells (10%) and MPPs (60%), while the lineage- c-Kit+ Sca-1- population represents committed progenitors. The accumulation of β-catenin in the progenitor compartment is in line with the block in granulocytic production observed in the dnTCF4 mouse model. However, even greater β-catenin increase was detected in the LSKs, which indicates that β-catenin might be regulating the EG response already at the level of stem cells and multipotent progenitors. However, the FACS analysis does not

have sufficient resolution to allow us to detect β-catenin activation in these rare populations. Nevertheless, our scRNA-seq provided further evidence regarding the activation of β-catenin in certain MPP sub-populations characterized by a myeloid/inflammatory identity rather than in the HSCs.

We observed that β-catenin activation occurs in a time-dependent manner in HSPCs upon LPS stimulation *in vitro*. Analysis of publicly available RNA seq data of short term HSCs, long term HSCs and MPPs stimulated with LPS and Pam3CSK4 for various timepoints *in vitro* showed increase in *Ctnnb1* mRNA, coding for β-catenin, as early as 2H after stimulation [142]. However, this might not be fully relevant, given, that β-catenin is regulated at protein level. In our experiments assessing the active form of β-catenin, we observed a modest activation 2H after stimulation *in vitro* while at 4H the active β-catenin levels reached a peak. Further, we explored the effect of both bacterial activators separately, and unlike LPS, Pam3CSK4 did not induce an increase in active β-catenin. Therefore, we concluded that the activation is LPS/TLR4 specific as TLR2/1 ligand Pam3CSK4 failed to induce β-catenin activation. This *in vitro* assay also allowed us to investigate whether β-catenin activation required pathogen recognition in a direct or indirect manner, since the *in vitro* settings are lacking the components that form the BM niche. We observed that β-catenin signaling activation can occur in a niche independent manner, however, we cannot not exclude that *in vivo* the BM niche further enhances β-catenin activation. Future studies blocking pathogen recognition specifically in the hematopoietic or the niche compartment will need to be performed to clarify this question.

To better understand the EG response, we analyzed the changes that occur early after induction of EG in HSPCs using scRNA-seq 4H after LPS injection, a timepoint, that was selected based on multiple criteria (granulocytic response, β-catenin activation and expression of EG marker genes). The gene expression analysis following LPS stimulation was already performed by De Laval who studied the basis of enhanced response following secondary LPS stimulus and Mann who was interested in myeloid skewing during aging [42], [142]. The analysis done by De Laval was done 24H after LPS stimulation using bulk RNA sequencing. We believe that 24H is too late to catch the most important changes that might be happening, as new granulocytes are already produced. The use of scRNA-seq, instead of bulk RNA sequencing, also allows us to understand the dynamics that happens at early timepoints of LPS treatment and allows us to detect subtle changes

in individual clusters, that might not be detected using bulk RNA sequencing. Similar to us, Mann *et al*. focused on earlier timepoints, from 30 minutes to 12 hours, however they investigated the effects of *in vitro* stimulation. As mentioned earlier, the *in vitro* settings lack the niche component and therefore might not be true representation of the EG response, where the niche plays an important role [34]. They performed both bulk and scRNA-seq, however because of multiple populations and conditions they only analyzed less than 150 cells per population using the scRNA-seq, which is very little to capture sub-population adaptations. Given all this we believe that our set up can bring valuable information that will help understand EG response. It would however be interesting to compare these data sets in the future, to elucidate the differences that the niche component and the time selection has on the EG response.

The hematopoietic hierarchy and lineage development in the HSPC compartment is a puzzling topic that was investigated by number of groups using different methods. In our scRNA-seq we were able to identify 10 clusters in each sample. As expected, GSEA revealed signatures of MMP 2, 3 and 4 subpopulations in the MPPs. More surprisingly, we were able to identify the MPP signatures even in the HSCs. While these MPP signatures in individual sub-populations in the MPP sample were quite strong and supported by GO analysis, the signatures were weaker in the HSC sample and often showed enrichment in the HSC homeostasis gene set. Similar to the MPP subpopulations, the existence of lineage-biased stem cells was observed before. Existence of myeloid biased, lymphoid biased and balanced HSCs, based on their output of mature cells during transplantation was reported previously [143]–[145]. Sanjuan-Pla *et al*. suggested that platelet-primed HSC reside at the top of the hematopoietic tree, but can still give rise to myeloid and lymphoid cells [20]. Further, Yamamoto *et al*. showed that there are two distinct populations amongst HSCs that can give rise either to megakaryocytes (megakaryocyte repopulating progenitors) or megakaryocytes and erythrocytes (megakaryocyte-erythrocyte repopulating progenitors). Additionally, they identified a population of common myeloid repopulating progenitors in the HSC compartment [19]. Altogether this indicates that both the HSCs and MPPs are heterogenous compartments that are composed of cells that already possess some level of lineage-bias and might differ by the ability to differentiate into other lineages.

Another interesting observation derived from our scRNA-seq is that the cluster composition of the MPP sample remained relatively similar between PBS and LPS conditions, while the HSC sample showed dramatic changes once stimulated with LPS. During the last years, emerging evidence has suggested that MPPs are responsible for daily supplies required during steady state hematopoiesis [146], [147]. On the contrary, in stress conditions alternative developmental routes are created, which position the HSC as the key population to cope with increased hematopoietic demands. During acute inflammation the platelets are replenished from a stem cell population that is characterized by stem-like, megakaryocyte-committed progenitor features [31]. Similarly, erythropoiesis is driven by HSCs, not MPPs during chronic erythroid stress [148]. On the other hand, recovery of the hematopoietic system is carried out by different MPP sub-populations which are overproduced from the HSCs following irradiation [26]. Our scRNA-seq data indicates that EG is mainly driven by drastic changes occurring in the HSCs, however MPPs are the first to respond to the stimulus by enhancing granulopoiesis in the already present myeloid-biased cluster.

In our settings, the majority of the clusters in both MPP and HSC samples carry megakaryocyte/erythroid signatures. This is in agreement with previously published findings that HSC that reside at the top of the hematopoietic tree have megakaryocytic signature and preferentially give rise to platelets but can also differentiate into myeloid or lymphoid cells [20]. Indeed, we did observe expression of *Vwf*, marker of the megakaryocytic HSCs, in our megakaryocytic clusters in the HSC sample. Interestingly, composition of all the Meg/E clusters was relatively unchanged between the PBS and LPS sample and no major inflammatory changes have been detected in these clusters. The only exception was cluster 7 in the MPP sample, that seemed to be LPS specific. Since this cluster also showed enrichment for the genes connected to response to type I interferon as well as HSC homeostasis, we hypothesize that this population might be similar to the stem-like, megakaryocytic progenitors that get activated during stress situations, as mentioned earlier. Indeed, we did find increased expression of *Itg2b* (CD41), a marker of the population identified by Haas *et al.* [31]. Upregulation of this gene was also identified in the megakaryocytic clusters in the HSC sample, suggesting that they might also contribute to the emergency megakaryopoiesis response.

Very little has been published about lymphoid-primed HSCs, however lymphoid primed multipotent progenitors that strongly inhibit the megakaryocyte/erythroid signature genes and upregulate early lymphoid genes have been identified [149]. We have identified 5 clusters with MPP4/lymphoid potential, and indeed they exhibit downregulation of the Meg/E signature. Nevertheless, most of these MPP4/lymphoid clusters did not show significant upregulation of the lymphoid GO terms, but instead showed enrichment in the HSC homeostasis gene set, suggesting prevalence of rather stem cell features. On the contrary, the MPP4/lymphoid sub-population in the MPP sample showed a strong lymphoid signature, suggesting that this is the cluster further committed to lymphoid development. Interestingly, this sub-population was PBS specific and was almost completely absent in the LPS sample. This indicates that inhibition of lymphopoiesis and favoring of myelopoiesis during EG is already present at the level of stem cells and multipotent progenitors [61].

In the steady state (PBS) condition we have identified only one myeloid-biased cluster that was formed of relatively low number of cells, however the myeloid signature got enriched in several sub-populations following LPS stimulation. Chen *et al*. had previously reported the existence of a myeloid biased HSC pool, which is marked by the expression of *Hdc,* increased expression of TLR4, and is important for increased production of myeloid cells during stress condition [150]. Indeed, our scRNA-seq showed that myeloid clusters upregulated *Hdc* expression*,* but not *Tlr4*. Interestingly, two out of three of the myeloid-biased HSC clusters also showed a lymphoid signature. Based on this and their similarity and closeness on the UMAP plot, we hypothesize that these cluster originate from the cells that were originally forming lymphoid clusters (3 and 6). Once we injected the animals with LPS, the myeloid signature was turned on in these cells in order to fight the infection. Indeed, the Passegué group had reported myeloid reprogramming of the MPP4 population to occur during regeneration following irradiation or 5-FU treatment [26], [58]. Similarly, in our scRNA-seq data we speculate that the MPP4 population was transcriptionally rewired to give rise mostly myeloid cells.

We further investigated the myeloid cluster that was already present in steady state conditions and showed enlargement following LPS stimulation in the MPP sample. Interestingly, this cluster showed enrichment of granulocytic markers, suggesting this might be the first source of granulocytes during EG response and that this population is

expanding at T = 4 hours after LPS stimulation. We found upregulation of 5 pathways including PLK-1, TNF, Aurora A and B and ceramide signaling in this MPP sub-population. Ceramide signaling has been linked to G-CSF signaling in neutrophils [151]. PLK-1 is a serin/threonine kinase that is important for cell-cycle regulation [152] and it was reported to be important in regulation of TNFα production and signaling following TLR stimulation [153], [154]. TNFα was shown to contribute to EG response in cancer settings [155], however whether it is important for non-cancerous EG response is yet to be elucidated. By far the most interesting for us was the enrichment in two Aurora kinases, that are important for cell proliferation [156], because Aurora kinase A has been identified as a β-catenin target gene in multiply myeloma [137]. Indeed, we found significant enrichment of β-catenin target genes in this granulocytic cluster. Nevertheless, as mentioned earlier, β-catenin target genes are not well described and how distinct target genes are critical amongst different tissues is not known. This is one of the main limitations of our study and we are aware that these genes might not be important for the hematopoietic system. This opens the question whether our findings are relevant in the hematopoietic system, but unfortunately, we are limited to the only datasets available in the GSEA database. Alternatively, we could try to compare our data with RNAseq of dnTCF4 HSCs that we have done previously [134], to investigate whether the genes that are employed during EG response are the ones that are regulated by β-catenin in the hematopoietic compartment.

One of the things that are not well described are the upstream activators of β-catenin signaling in different settings. Thus, we screened our data for Wnt ligands and to our surprise we found a significant increase of *Wnt10b* in lymphoid/myeloid cluster 9 in the HSC sample. We validated these findings *in vitro* by stimulating c-Kit+ cells with LPS. *Wnt10b* mRNA was increased following LPS stimulation and was coinciding with the upregulation of *Cebpb* and β-catenin activation. The production of Wnt10b during times of regeneration has been shown before following irradiation and 5-FU treatment [118], [119], [127]. It was reported previously that Wnt10b is produced by hematopoietic cells and can activate β-catenin signaling in HSPCs. Indeed, we detected an increase in active β-catenin levels following *in vitro* stimulation with Wnt10b, suggesting that Wnt10b is an upstream activator of β-catenin signaling in HSPCs. Based on our findings and the published observations we believe that Wnt10b is important for the EG response. However, the expression of Wnt10b that we observed might not be sufficient to drive EG

response. Based on published results, Wnt10b is produced mostly hematopoietic cells in steady state, while during stress and regeneration it is produced mostly by cells of the BM niche, in particular endothelial cells [118], [119], [127]. Therefore, it is possible that this also occurs during EG and that Wnt10b production might be further supported by the niche. We aim to investigate the role of Wnt10b in steady state and emergency granulopoiesis in the near future to understand the impact the Wnt10b/β-catenin/TCF/LEF axis has on the EG response.

In conclusion, we believe that in our scRNA-seq data we caught the very initiation of the EG response. At the level of MPPs we observed accumulation of myeloid cells from a cluster that was already present in steady state, as well as emergence of two new clusters with inflammatory signature. Ever more striking were the changes in the HSCs where we believe we caught the exact timepoint when some of these cells are gaining myeloid signature while losing the lymphoid signature as proposed in Figure 14. However, it is important to keep in mind that scRNA-seq analysis is a very complex process that requires a lot of time and bioinformatics skills. The data presented in this thesis represent just our preliminary analysis of the results. We are aware that a deeper and more complex analysis will need to be performed to complete our interpretations and conclusions. Nevertheless, our data brings an insight into the early population dynamics in the HSPC compartment following LPS stimulation and shows that the emergency response is carried out by both the HSC and MPP compartments. This further supports the plasticity of these compartments and their alternative regulation in different stress conditions.

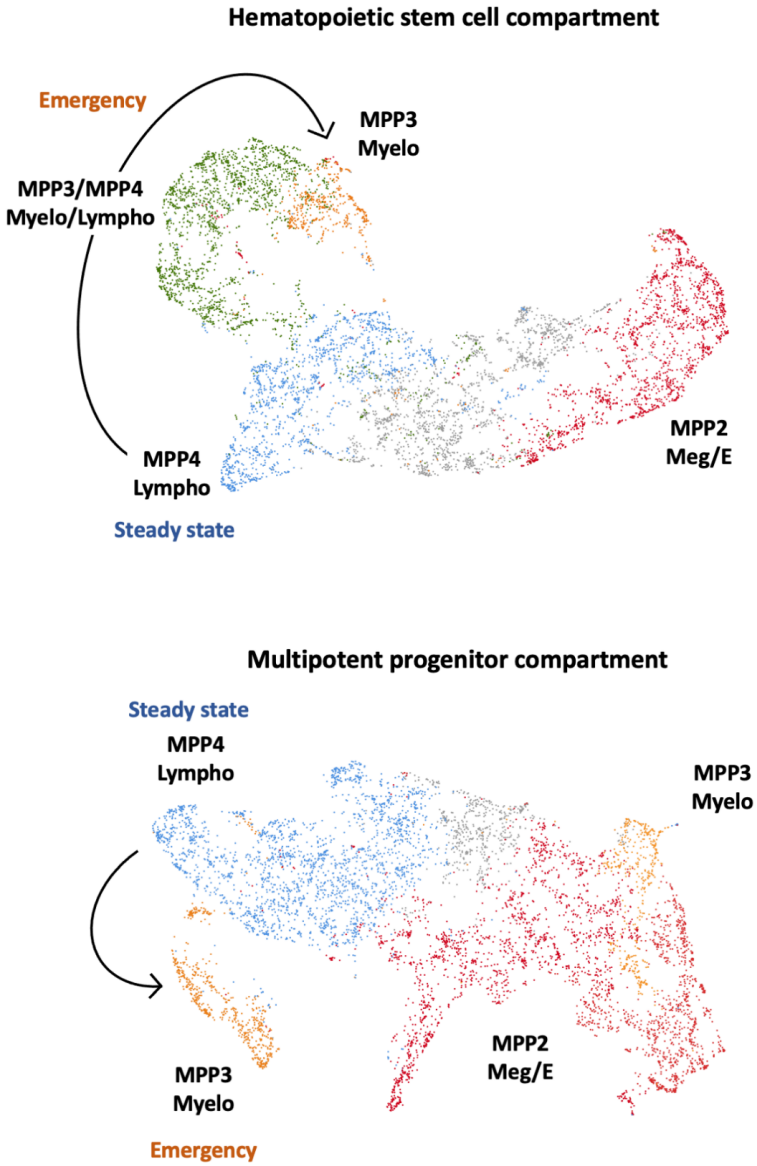

Figure 14. Proposed way of myeloid lineage commitment from lymphoid biased stem cells (top) or progenitors (bottom) during emergency conditions.

10 Conclusion

In conclusion, we identified β-catenin-TCF/LEF signaling complex as a crucial signaling pathway employed during emergency granulopoiesis. β-catenin signaling is necessary during myeloid progenitor expansion and skewing towards granulocytes, critical steps of EG. In addition to that, we report that β-catenin is activated at early stages of EG, and its activation is at least partially niche independent and restricted to LPS stimulation, not Pam3CSK4. Further, we demonstrated that scRNA-seq performed at early times after LPS administration is a valid method to understand the rewiring from SSG to EG at different levels, i.e. single cell level, sub-populations in HSCs and MPPs, signaling pathways, gene ontologies, and particular genes. The compilation of these observations provided us with a better understanding of the mechanisms occurring in HSPCs during early activation of EG. Our main observations include the identification of MPP and HSC sub-populations characterized by MPP2/3/4 profiles. Importantly, we conclude that initiation of EG demands changes in MPPs as well as in HSCs, and that new differentiation routes need to be originated from the HSC compartment to cope with the increased granulocytic demands. Remarkably, only one myeloid cluster was identified in steady state and it showed enrichment in granulocytic markers. This cluster was enlarged following LPS stimulation and was enriched for β-catenin target genes. Additionally, we identified Wnt10b, an upstream activator of β-catenin signaling, to be upregulated in one of the inflammatory clusters. Altogether, our data point at a critical role of the Wnt/β-catenin-TCF/LEF signaling pathway in activation of the EG program at the HSPC level at early stages upon acute infection.

11 References

* Marks review articles

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