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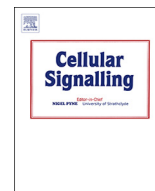
Divergent effects of Wnt5b on IL-3- and GM-CSF-induced myeloid differentiation

Marina Mastelaro de Rezende[a,b], John-Poul Ng-Blichfeldt[b,c], Giselle Zenker Justo[a,d],
Edgar Julian Paredes-Gamero[a,e], Reinoud Gosens[b,*]

[a] Departamento de Bioquímica, Universidade Federal de São Paulo (UNIFESP), São Paulo 04044-020, Brazil
[b] Department of Molecular Pharmacology, University of Groningen, Groningen 9713AV, Netherlands
[c] MRC Laboratory of Molecular Biology, Cambridge Biomedical Campus, Cambridge CB2 0QH, UK
[d] Departamento de Ciências Farmacêuticas, Universidade Federal de São Paulo (UNIFESP), Diadema 09913-030, Brazil
[e] Faculdade de Ciências Farmacêuticas, Alimentos e Nutrição, Universidade Federal de Mato Grosso do Sul, 79070-900, Campo Grande, Mato Grosso do Sul, Brazil

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ABSTRACT

The multiple specialized cell types of the hematopoietic system originate from differentiation of hematopoietic stem cells and progenitors (HSPC), which can generate both lymphoid and myeloid lineages. The myeloid lineage is preferentially maintained during ageing, but the mechanisms that contribute to this process are incompletely understood. Here, we studied the roles of Wnt5a and Wnt5b, ligands that have previously been linked to hematopoietic stem cell ageing and that are abundantly expressed by both hematopoietic progenitors and bone-marrow derived niche cells. Whereas Wnt5a had no major effects on primitive cell differentiation, Wnt5b had profound and divergent effects on cytokine-induced myeloid differentiation. Remarkably, while IL-3-mediated myeloid differentiation was largely repressed by Wnt5b, GM-CSF-induced myeloid differentiation was augmented. Furthermore, in the presence of IL-3, Wnt5b enhanced HSPC self-renewal, whereas in the presence of GM-CSF, Wnt5b accelerated differentiation, leading to progenitor cell exhaustion. Our results highlight discrepancies between IL-3 and GM-CSF, and reveal novel effects of Wnt5b on the hematopoietic system.

1. Introduction

Effective immunity and tissue homeostasis throughout life depend on the ability of hematopoietic stem cells and progenitors (HSPC) to generate a balanced supply of the specialized lymphoid and myeloid cell types that constitute the hematopoietic system [53]. Myeloid lineage commitment initiates from the common myeloid progenitor, which differentiates to give rise to critical components of the innate immune system including granulocytes, monocytes and mast cells, in addition to platelets and erythrocytes [53]. Recent studies indicate that the myeloid lineage is preferentially maintained over the lymphoid lineage during ageing [14,19,21,37] and this so-called myeloid skewing is of interest as it may contribute to the development of chronic diseases associated with ageing. In this context, it is of great interest to understand how the molecular and microenvironmental changes that occur during ageing impact on myeloid differentiation as this may yield potential therapeutic targets.

Myeloid lineage development is regulated by a complex network of molecules. Of central importance are cytokines and growth factors

produced by the bone marrow microenvironment [6,24,44,64], which comprises numerous cells types of both hematopoietic origin, including monocytes and lymphocytes, and non-hematopoietic origin, including fibroblasts, adipocytes and endothelial cells [3,24,68]. Interleukin-3 (IL-3) and granulocyte macrophage-colony stimulating factor (GM-CSF), produced by monocytes and lymphocytes, have particularly crucial roles in myeloid differentiation [24,64]; GM-CSF is also produced by non-hematopoietic bone marrow cells [3,6,52]. IL-3 and GM-CSF have overlapping activities [9,44,47], including regulating proliferation and differentiation of myeloid progenitors [2,44], stimulation of granulocyte-monocyte-biased colony formation in colony formation unit (CFU) assay [47] and activation of granulocytes and monocytes [2,9,44]. In primitive hematopoietic cells, similar intracellular proteins are recruited to generate the cited outcomes, such as PLCγ2, calmodulin kinase and MEK/ERK signaling [35], although PKC activation is not observed after GM-CSF treatment and the pattern of activation of MEK/ERK varies [35].

Primitive cells including HSCs (hematopoietic stem cells) and multipotent progenitors are more sensitive to IL-3, which supports stem cell

maintenance [15–17], whilst committed myeloid progenitors are more sensitive to GM-CSF, which drives differentiation and proliferation [15–17].

Numerous Wnt signaling components have been identified within the bone marrow microenvironment [57,63,66], and some of these appear to play significant roles in hematopoietic ageing [19,32,36]. Wnt ligands induce intracellular signaling events by binding to cell surface frizzled (Fzd) receptors, stimulating either β-catenin dependent (canonical) or independent (non-canonical) signaling pathways [51]; non-canonical Wnt signaling can also be mediated by interactions with cell surface receptor tyrosine kinases of the Ror and Ryk families [25].

The Wnt5 subfamily of Wnt ligands is of particular interest as both Wnt5a and Wnt5b are expressed by primitive hematopoietic cells [8,50,54,55] and niche cells [19,41,42,48,54,58,71]. Wnt5a and Wnt5b are structurally related with high amino acid sequence similarity [31]. However, Wnt5a and Wnt5b are expressed in non-overlapping patterns during mouse development [20], and functional differences have been described for Wnt5a and Wnt5b in regulation of chondrocyte differentiation [72], and mammary epithelial cell growth [31].

Wnt5a and Wnt5b are expressed during embryonic hematopoietic initiation [12], and regulate hematopoietic cell fate during adult blood homeostasis [12,19]. Importantly, recent studies revealed that Wnt5a expression by HSCs increases with age, leading to augmented hallmarks of hematopoietic senescence [19] and to imbalances in Notch and calcium pathways [21].

Altogether, these studies led us to hypothesize that Wnt5a and Wnt5b contribute to age-related increases in cytokine-driven myeloid differentiation. We thus investigated the role of Wnt5a and Wnt5b in cytokine-induced HSPC myeloid differentiation using an in vitro colony formation unit (CFU) assay [45], with IL-3 and GM-CSF treatment to stimulate differentiation down myeloid lineages. We found that Wnt5a and Wnt5b had profound and divergent effects on cytokine-induced myeloid differentiation. Remarkably, while IL-3-mediated myeloid differentiation was largely repressed by Wnt5b, GM-CSF-induced myeloid differentiation was augmented. Furthermore, in the presence of IL-3, Wnt5b enhanced HSPC self-renewal, whereas in the presence of GM-CSF, Wnt5b accelerated differentiation, leading to progenitor cell exhaustion. Our results highlight discrepancies between IL-3 and GM-CSF, and reveal novel effects of Wnt5b on the hematopoietic system.

2. Methods

2.1. Animal models

Adult (2 to 4 months old) C57Bl/6 J specified pathogen-free mice of both sexes were used for this study. Mice were housed at the UMCG (University Medical Center Groningen, The Netherlands) Central Animal Facility and CEDEME (Centro de Desenvolvimento de Modelos Experimentais para Biologia e Medicina, from UNIFESP – Universidade Federal de São Paulo, Brazil) and kept in a controlled habitat under a 12/12 h dark-light cycle, with food and water ad libitum. All experimental procedures followed ethical research guidelines and were approved by ethical committees in both institutions (AVD105002015303 and 1,522,060,515, for University of Groningen and UNIFESP, respectively).

2.2. Hematopoietic cell extraction

To obtain hematopoietic cells, animals were euthanized by cervical dislocation or by deep anesthesia using ketamine/dexdomitor following rapid exsanguination via the abdominal aorta. Afterwards, femurs were collected. Femoral content was flushed out with a syringe filled with IMDM medium and homogenized. The homogenate was incubated at 37 °C for 2 h in order to reduce the presence of adherent mononuclear cells in the supernatant. Afterwards, the supernatant was separated; cells were counted and used for experiments.

2.3. Colony formation units assay (CFU) and replating

We investigated hematopoietic progenitor potential using the colony forming unit (CFU) assay, in which 5×10^4 bone marrow cells were seeded into a semi-solid medium (MethoCult M3134, StemCell Technologies – Vancouver, Canada) and the number of resulting colonies are counted manually using a light microscope at $10\times$ magnification [45]. Cells were plated in 1 mL of this medium supplemented with 10% FBS, 0,1% bovine serum albumin and 1% penicillin/streptomycin and incubated at 37 °C and 5% CO_2 for 2 weeks for the first round of colony reading. The treatments consisted of: IL-3 (10 ng/mL), IL-3 (10 ng/mL) + Wnt5a (50 and 200 ng/mL representing EC50 and submaximal response in other cell systems, respectively [4], IL-3 (10 ng/mL) + Wnt5b (50 and 200 ng/mL). The same co-treatments were done using GM-CSF (10 ng/mL). Cytokines (403-ML and 415-ML for IL-3 and GM-CSF, respectively) and Wnt ligands (references 645-WN and 7347-WN for Wnt5a and 5b, respectively) were purchased from R&D systems (Minneapolis, USA). AS control for β-catenin signaling activation, CHIR99021 (2 μM) was used as well, in the presence of IL-3 and GM-CSF.

After 2-weeks of incubation, there was a first round of colony reading. Colonies were counted according to subtype and areas of colonies were measured. After this, colonies were dissociated (after centrifugation and resuspension) and counted (for the calculation of cells by colony ratio and total number of cells per plate). For the replating assay, after dissociation and counting, cells were plated back into semi-solid medium for two more weeks and this was subsequently repeated until colonies were no longer formed. The treatments used previously were repeated. Half-way during the first round, directly after the first round and after the second round of reading, some plates were used for gene expression and flow cytometry analysis (Fig. 1).

After the first round of colony reading, for some experiments, plates passed through mechanical separation of colonies by subtype and the replating was done for each subtype individually. After replating, cells were again incubated for 2 weeks and read thereafter.

After 2 weeks, some colonies were also isolated by subtype and replated, differently of the previously described, in which all colonies were replated together.

2.4. Liquid culture (LC)

Cells were incubated in IMDM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 10^{-6} M hydrocortisone for 1 week, and treated with IL-3/GM-CSF in the absence and presence of Wnt5b. The concentration of cells used for this assay was 1×10^6 cells/mL. We used the same starting population as before to mirror the population used in the CFU assay. After 1 week, cells were washed, counted and analyzed by flow cytometry.

2.5. Flow cytometry

Cells acquired from the CFU and LC assays were analyzed by flow cytometry to provide information about primitive cell maintenance, differentiation and cell death. All cells were stained in PBS with 0,1% bovine serum albumin and incubated at room temperature protected from light for 20–40 min prior to flow cytometry reading, except for cell death experiments, in which annexin buffer (pH 7.4) was used (0.1 M Hepes, 1.4 M NaCl, and 25 mM $CaCl_2$).

CFU cells were used after 1, 2 and 4 weeks of incubation and the panel used for primitive cells maintenance and cell death was: Annexin V-FITC, Lin cocktail-PE (B220, CD3e, CD11b, TER119 and Ly-6-G/C), 7-AAD and c-Kit-APC (CD117) and for differentiation, Gr-1-FITC (Ly-6G/Ly-6C), B220-PE, Mac-1- PECy7 (CD11b) and c-Kit-APC (CD117). For LC cells, panels used were: for primitive cells maintenance, Lin-PE cocktail, Sca-1-PECy7 (Ly-6A/E) and c-Kit-APC (CD117), for differentiation, F4/80-FITC, Gr-1-PE, Mac-1-PECy7 and TER119-APC, and for
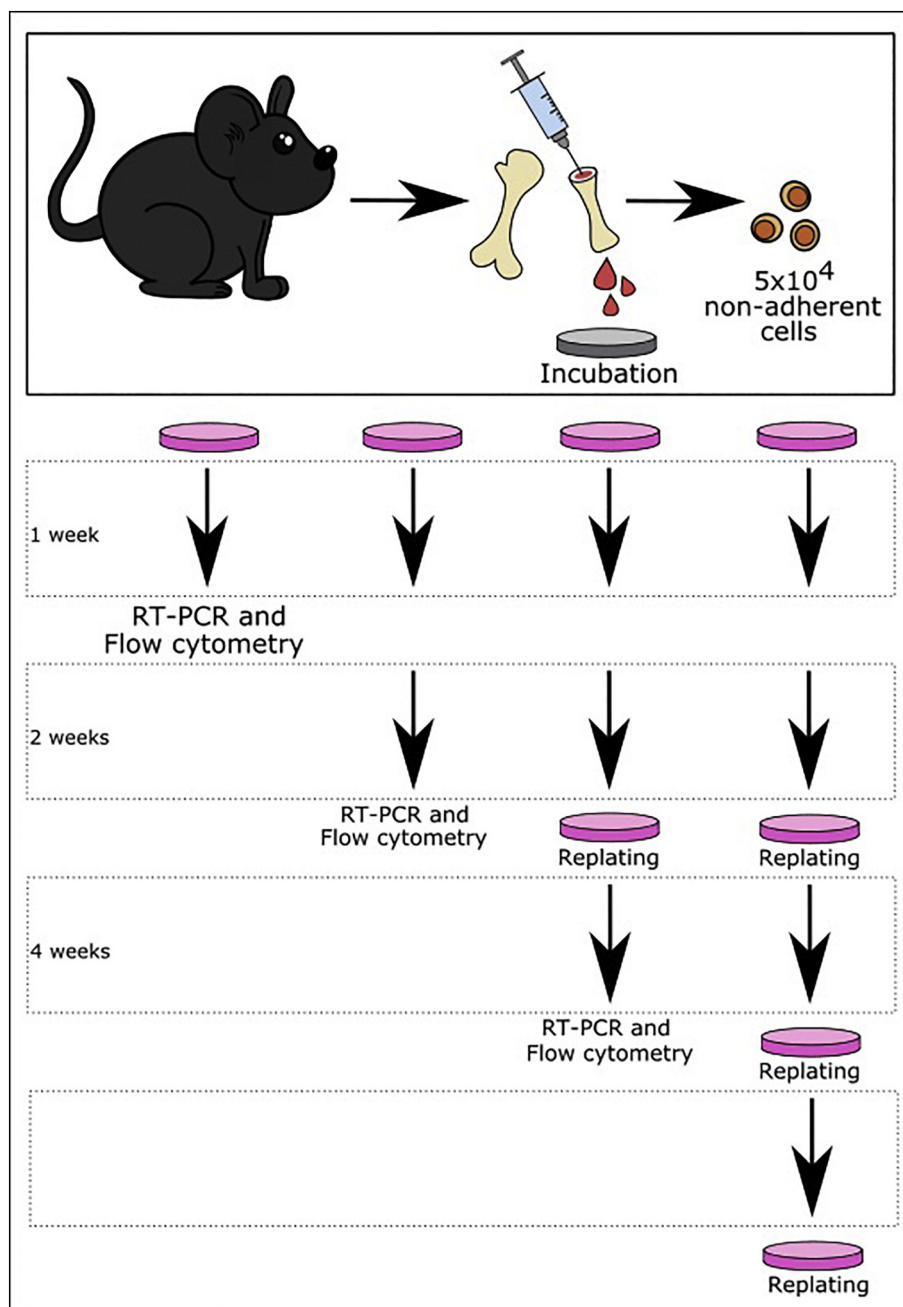
Fig. 1. Experimental design.

cell death, Annexin V-FITC, Lin-PE cocktail, 7-AAD and c-Kit-APC. All antibodies used were purchased from BD Biosciences and equipment used was Accuri C6, from BD Biosciences as well (Michigan, USA).

Flow cytometry was used to semi-quantify GSK3β phosphorylation after IL-3 and GM-CSF treatment as well. For this, bone marrow-derived cells were incubated with the respective cytokine for 15, 20 and 30 min at 37 °C and agitation, followed by fixation with 2% paraformaldehyde (Becton Dickinson) for 15, 20 and 30 min, washing with 0.1 M glycine and permeabilization with 0.001% triton X-100. Primary antibody labeling was done using rabbit p-GSK3β (#5558, Cell Signaling) for 2 h and secondary, goat Anti-rabbit Alexa Fluor 488 (Invitrogen) for another 2 h. p-GSK3β phosphorylation was calculated as the ratio of the geometric mean of histogram fluorescence signal between treated and untreated samples. The same panel of conjugated antibodies used for primitive cell maintenance after LC was used here as well. For HSPC population gating, we used the Lin⁻Sca-1⁺c-Kit⁺ immunophenotype,

whereas for progenitor population, Lin⁻ Sca-1⁻c-Kit⁺ was used. Same Lin cocktail was used in CFU assay samples, together with viability markers Annexin V and 7AAD. The gating strategy is also represented in Supplementary Fig. 1.

2.6. RT-PCR

Cells were harvested at the indicated time points after the CFU assay, washed and dissociated in PBS. Total RNA was then isolated using Trizol (ThermoFisher Scientific, Massachusetts, USA) according to the manufacturer's instruction. The total RNA concentration was measured using the NanoDrop ND1000 spectrophotometer (Thermo Scientific, Wilmington, USA). Total RNA was reverse transcribed using the Reverse Transcription System (Promega; Madison, USA). For RT-PCR, cDNA was combined with FastStart Universal SYBR Green Master Mix (Roche Applied Science; Penzberg, Germany) and specific primer

sets (Biolegio; Nijmegen, the Netherlands) using the Eco Personal qPCR system (Illumina, California, USA). Primer sequences are listed in Supplementary Table 1. The qPCR protocol started with activation at 95 °C for 15 min, followed by 45 cycles of denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s and elongation at 72 °C for 30 s. A final elongation step of 5 min at 72 °C was added at the end of the protocol. Data was analyzed using LinRegPCR software and results were expressed as ratio of the starting concentration (N0) of each gene of interest corrected to the geometric mean of the N0 value of 2 reference genes (GAPDH and β-actin).

2.7. Statistics

Data are shown as means ± SEM, except for colony area measurements, for which the median (median is usually presented with interquartile range) is shown. Statistical differences between means were evaluated by a Student's *t*-test or a Mann-Whitney *U* test, in case of comparisons between two groups, as appropriate. All experiments were repeated with cells obtained from at least 3 animals. Differences were considered significant when $p < .05$.

3. Results

3.1. Divergent effects of Wnt5a and Wnt5b on IL-3- and GM-CSF-induced myeloid differentiation

We investigated the roles of Wnt5a and Wnt5b on HSPC potential using an in vitro colony formation unit (CFU) assay, with IL-3 and GM-CSF treatment to stimulate differentiation down myeloid lineages. Specific differentiation outcomes were determined by formation of GM-CFU (granulocyte-monocyte colony formation units), M-CFU (monocyte colony formation units) or G-CFU (granulocyte colony formation units). Between these types of colonies, GM-CFU is the most primitive subtype and mostly formed by common myeloid progenitors and granulocyte-macrophage progenitors [26,30,38]. A characteristic of its higher degree of primitiveness is the bipotency observed, whereas M and G-CFU are unipotent [38]. In the presence of IL-3, Wnt5a was unable to affect colony formation at either 50 ng/mL or 200 ng/mL (Fig. 2A–D). Wnt5b, on the other hand, inhibited colony formation at the higher concentration, affecting mainly the GM and M subtypes (GM-CFU: 11.50 ± 2.6 for IL-3 and 3.33 ± 1.4 for IL-3 + Wnt5b; M-CFU: 33.88 ± 3.5 for IL-3 and 18.33 ± 1.8 for IL-3 + Wnt5b, $p < .05$, Figs. 2A–D). The same batches of Wnt5a were used in parallel experiments and were found to repress lung organoid formation, confirming its biological activity.

In the presence of GM-CSF, Wnt5a had no effect on total myeloid colony number or number of specific colony subtypes (Fig. 2E). In contrast, Wnt5b significantly increased total number of colonies formed (72.88 ± 4.6 for GM-CSF and 112.50 ± 2.3 for GM-CSF + Wnt5b – 200 ng/mL, $p < .05$, Fig. 2E). This was attributed to increased number of M and G-CFU subtype colonies (M-CFU: 45.50 ± 6.8 for GM-CSF, 70.86 ± 10.1 for GM-CSF + Wnt5b – 50 ng/mL, 80.17 ± 2.0 for GM-CSF + Wnt5b – 200 ng/mL; G-CFU: 12.50 ± 2.2 for GM-CSF and 20.67 ± 1.3 for GM-CSF + Wnt5b – 200 ng/mL, all $p < .05$, Fig. 2F–H). Interestingly, a significant decrease in the GM-CSF colony subtype was observed at the lowest concentration (14.00 ± 2.5 for GM-CSF and 7.00 ± 1.1 for GM-CSF + Wnt5b – 50 ng/mL; $p < .05$, Fig. 2F). These data indicate substantial differences between Wnt5a and -b in regulating myelopoiesis, and show that Wnt5b has divergent effects on myelopoiesis induced by IL-3 and GM-CSF.

3.2. IL-3 and GM-CSF have similar effects on myeloid differentiation

As previously discussed, there are similarities between IL-3 and GM-CSF intracellular signaling [29,35,56,60], what do not is in seeming contrast to the differential Wnt5b effects on these contexts and in the

promotion of myeloid differentiation. In view of the observed differences in concerted action with Wnt5b, we decided to compare the cell populations obtained with IL-3 and GM-CSF in more detail. Even though it is well-described how similar the outcomes of the IL-3 and GM-CSF treatment are, we aimed to assure that the observed differences in the Wnt5b effects were not caused by any experimental differences in IL-3 and GM-CSF themselves. IL-3 and GM-CSF produced very similar total myeloid colony numbers (Fig. 3A) and had similar effects on the colony subtype distribution for GM-CFU, M-CFU or G-CFU. A similar result was obtained for colony size (Fig. 3B).

Colonies were then replated to address effects on the progenitor cell population and their potential. As shown in Fig. 3C and D, the colony forming ability was maintained slightly longer in the presence of IL-3 than in the presence of GM-CSF (half-life = 1.4 rounds for IL-3, and 1.0 round for GM-CSF, Fig. 3C and D – this difference was not statistically significant), whereas the average number of cells per colony after the first CFU round was not different between IL-3 and GM-CSF (Fig. 3E). The liquid culture assay showed no major difference either, with very similar numbers of cells in the presence of IL-3 and GM-CSF (Fig. 3F).

IL-3 and GM-CSF-driven myelopoiesis was investigated further by analysis of cell surface marker expression. The relative proportion of committed (Lin$^+$) and non-committed cell (Lin$^-$) cell numbers observed after 1 week of liquid culture was similar for IL-3 and GM-CSF (Figs. 3G and H). However, the percentage of the primitive population Lin$^-$c-Kit$^+$ cells was maintained at higher levels with IL-3 than with GM-CSF (1.38 ± 0.4 in IL-3, 0.24 ± 0.2 in GM-CSF, $p < .05$, Fig. 3I). Further, when analyzing subpopulations from the CFU assay, we observed an initial increase in the number of Lin$^+$ cells compared to Lin$^-$ cells in response to Wnt5b and GM-CSF (Supplementary Fig. 3), whereas no Wnt5b influence is observed in presence of IL-3. A further analysis shows that Wnt5b promotes early apoptosis of Lin$^+$ cells in combination with GM-CSF, which is not observed in combination with IL-3 (Supplementary Fig. 3). Altogether, these data indicate that IL-3 and GM-CSF produce very similar outcomes, although IL-3 maintains the number of primitive cells better in comparison to GM-CSF. Interestingly, in response to IL-3, phosphorylation of GSK3β can be observed in (committed) progenitor cells (Prog - Lin$^-$Sca-1$^-$c-Kit$^+$ − the histograms are shown in Supplementary Fig. 2), which is not seen for GM-CSF (Fig. 3K). Although GSK3β phosphorylation is an indicative of pathway activation, we are aware of the need for more investigation for the mechanistic understanding of the observed differences. For these, we used bone marrow cells of the TCF/Lef:H2B-GFP fusion Wnt reporter mice [18], but no obvious GFP signal in bone marrow-derived cells was found and we could not detect overt localization of β-catenin protein using immunofluorescence microscopy, either at baseline or in response to cytokine stimulation. In line with these observations, in western analyses of lysates of non-adherent cells exposed to GM-CSF or IL-3 in the absence and presence of WNT-5B we were unable to detect changes in total β-catenin content and did not detect active (non-phospho) β-catenin expression (data not shown).

3.3. Wnt5b maintains primitive progenitors in the presence of IL-3

To further investigate the effects of Wnt5b on IL-3-driven myelopoiesis, and with this difference between IL-3 and GM-CSF in mind, the colonies formed in the presence of IL-3 and Wnt5b were dissociated and replated. With every round of replating, the number of colonies formed decreased (colony number half-life = 1.4 rounds for IL-3 alone) until after 5 rounds of replating, essentially no colonies were maintained (Fig. 4A). The number of colonies formed in the presence of IL-3 and Wnt5b was much higher in the second round of replating (a 6-fold difference compared to round 1–48.44 ± 4.5 for the first round and 329.67 ± 82.2 for the second, $p < .05$, Fig. 4B). In addition, the colony number half-life was twice as long in the presence of Wnt5b (2.9 rounds for IL-3 and Wnt5b, Figs. 4A and B; $p < .05$, two-way ANOVA).

To investigate whether the increased colony formation at round 2

IL-3
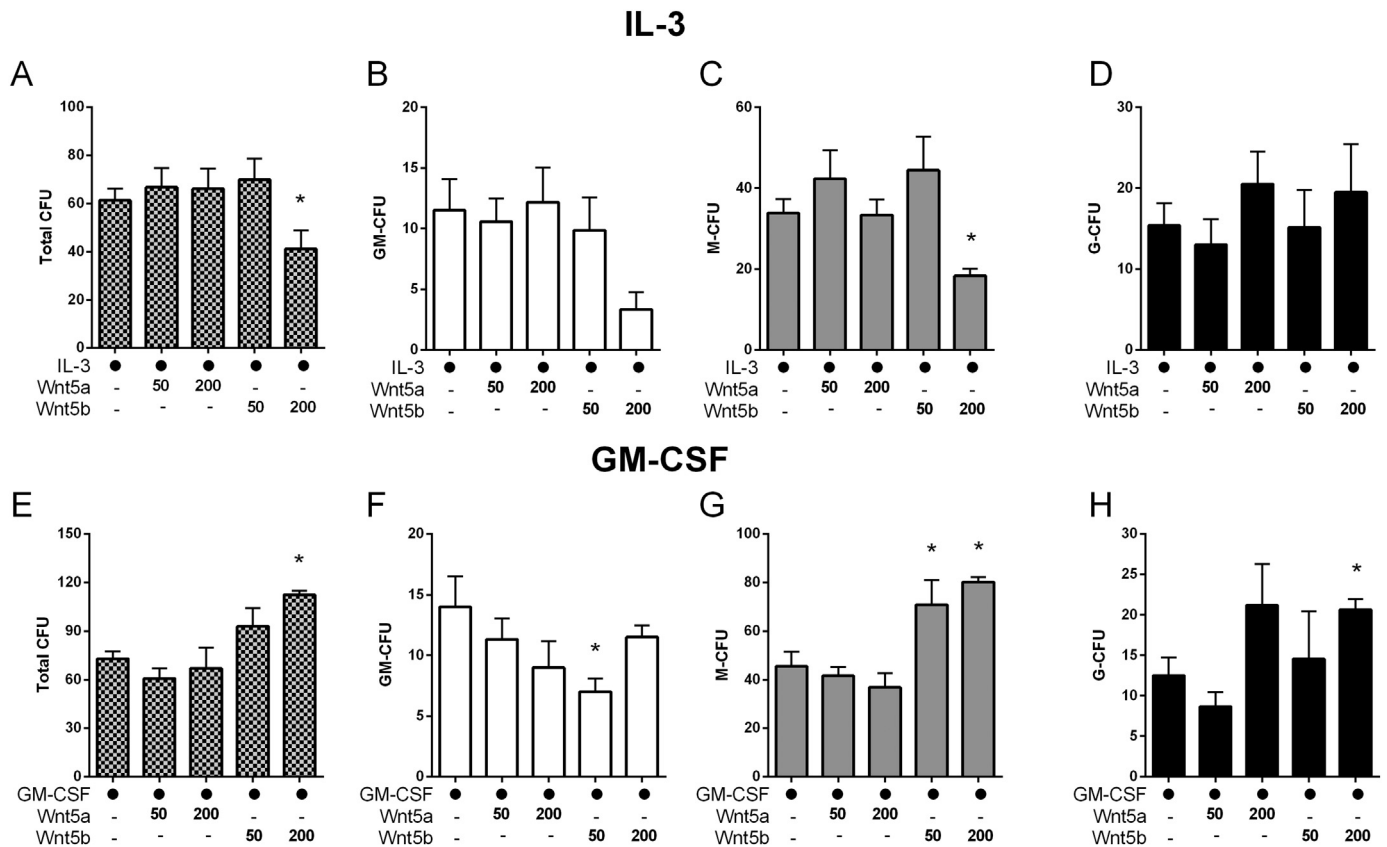
GM-CSF

Fig. 2. Effects of Wnt5a and Wnt5b on IL-3 and GM-CSF induced myeloid colony formation. A) Total numbers of colonies formed by IL-3 (10 ng/mL) in the absence and presence of Wnt5a (50 and 200 ng/mL) and Wnt5b (50 and 200 ng/mL). Panels B-D show differential data for B) GM-CFU; C) M-CFU and D) G-CFU. E) Total numbers of colonies formed by GM-CSF (10 ng/mL) in the absence and presence of Wnt5a and Wnt5b. Panels F–H show differential data for F) GM-CFU; G) M-CFU and H) G-CFU. Data are presented as mean $\pm$ SEM. *p < .05, n = 6–8.

was caused by an increase in the number of colony-forming cells, colony size was measured directly after the first round. Wnt5b did not influence colony size (Fig. 4C). Wnt5b did not affect the average number of cells per colony either (Fig. 4D). To check if the increased colony-forming potential was specifically maintained in a single sub-population, first analyzed the ratio between Lin$^+$ and Lin$^-$ cells after 1 week of CFU, but there was no change in this parameter in presence of Wnt5b (Supplementary Fig. 3A) and then, we replated isolated subtypes of colonies (G, GM and M) but found no differences between these colony subtypes either (Fig. 4E).No difference between populations, colonies sizes or number of cells by colony suggested that Wnt5b would influence functionality (such as potential maintenance or differentiation) of the hematopoietic cells, rather than induce proliferation and population imbalance. Thus, intracellular components were analyzed.

We therefore analyzed gene expression patterns of colonies treated with IL-3 and Wnt5b in more detail after 1 week and 2 weeks of treatment for general markers of cell cycle progression and differentiation. There was no difference in cell cycle markers after 1 week of culture – Figs. 4F and G). However Cdk1 expression increased and cy-clin D1 tended to increase after 2 weeks (Cdk1: 0.015 $\pm$ 0.0 for IL-3 and 0.034 $\pm$ 0.0 for IL-3 + Wnt5b, p < .05, Figs. 4H-I). The primitive cell markers GATA-2 and Ifitm-1 showed a similar pattern (Ifitm1: 3.035 $\pm$ 0.2 for IL-3 and 3.730 $\pm$ 0.6 for IL-3 + Wnt5b, p < .05, Fig. 4J-M). No effects on more mature markers (Irf8 and Gfi-1) were observed (not shown). No change in DKK-1, Axin2 or LRP5 gene expression was observed (Fig. 4O and N). To check how non-canonical Wnt signaling could be affected by Wnt5b treatment in presence of IL-3, we also analyzed gene expression of signature genes involved in non-canonical Wnt signaling. 3 genes were selected with known roles in noncanonical signaling, being c-Jun, c-Fos and Cdc42. As can be seen in

Fig. 4Q-T, the presence of Wnt5b in combination with IL-3 inhibited the expression of these genes.

3.4. Wnt5b induces committed progenitor activation and progenitor exhaustion in the presence of GM-CSF

We next investigated the effects of Wnt5b on GM-CSF-driven mye-lopoiesis. In sharp contrast to IL-3, Wnt5b had no effects whatsoever on the kinetics of decay of GM-CSF-induced colony number or subtype after replating (Fig. 5A and B), with colony number half-lives being identical (1.0 round for both conditions). Wnt5b did significantly de-crease average colony size compared to GM-CSF alone (6.8 × 10^5 $\pm$ 3.3 × 10^4 for GM-CSF and 5.1 × 10^5 $\pm$ 2.2 × 10^4 for GM-CSF + Wnt5b, p < .05, Fig. 5C). Wnt5b treatment had no effect on the average number of cells per colony (Fig. 5D). When replating in-dividual subtypes of colonies, Wnt5b decreased the number of GM-CFU and M-CFU (GM-CFU: 7.21 $\pm$ 0.7 for GM-CSF and 3.33 $\pm$ 0.7 for GM-CSF for GM-CSF + Wnt5bp < .05; M-CFU: 6.00 $\pm$ 1.1 for GM-CSF and 2.90 $\pm$ 0.5 for GM-CSF + Wnt5b, p < .05, Fig. 5E).

In contrast to what was observed for the presence of IL-3, in the presence of GM-CSF, Wnt5b did not have any effect on gene expression of Cdk1 or CyclinD1 after 2 weeks of the CFU assay (Fig. 5G and H), or at any other time point (data not shown). There was no difference in the primitive cell markers GATA-2 and Ifitm-1 (Fig. 5I and J). These data highlight the possibility that Wnt5b acts on committed progenitors in the presence of GM-CSF. We therefore investigated cell surface marker expression in week 1 and 2 of the CFU assay. Interestingly, Wnt5b significantly increased the ratio of Lin$^+$/Lin$^-$ cells after 1 week of CFU assay (0.87 $\pm$ 0.1 for GM-CSF and 1.77 $\pm$ 0.3 for GM-CSF + Wnt5b, p < .05, Fig. 5F), indicating higher presence of committed progenitors
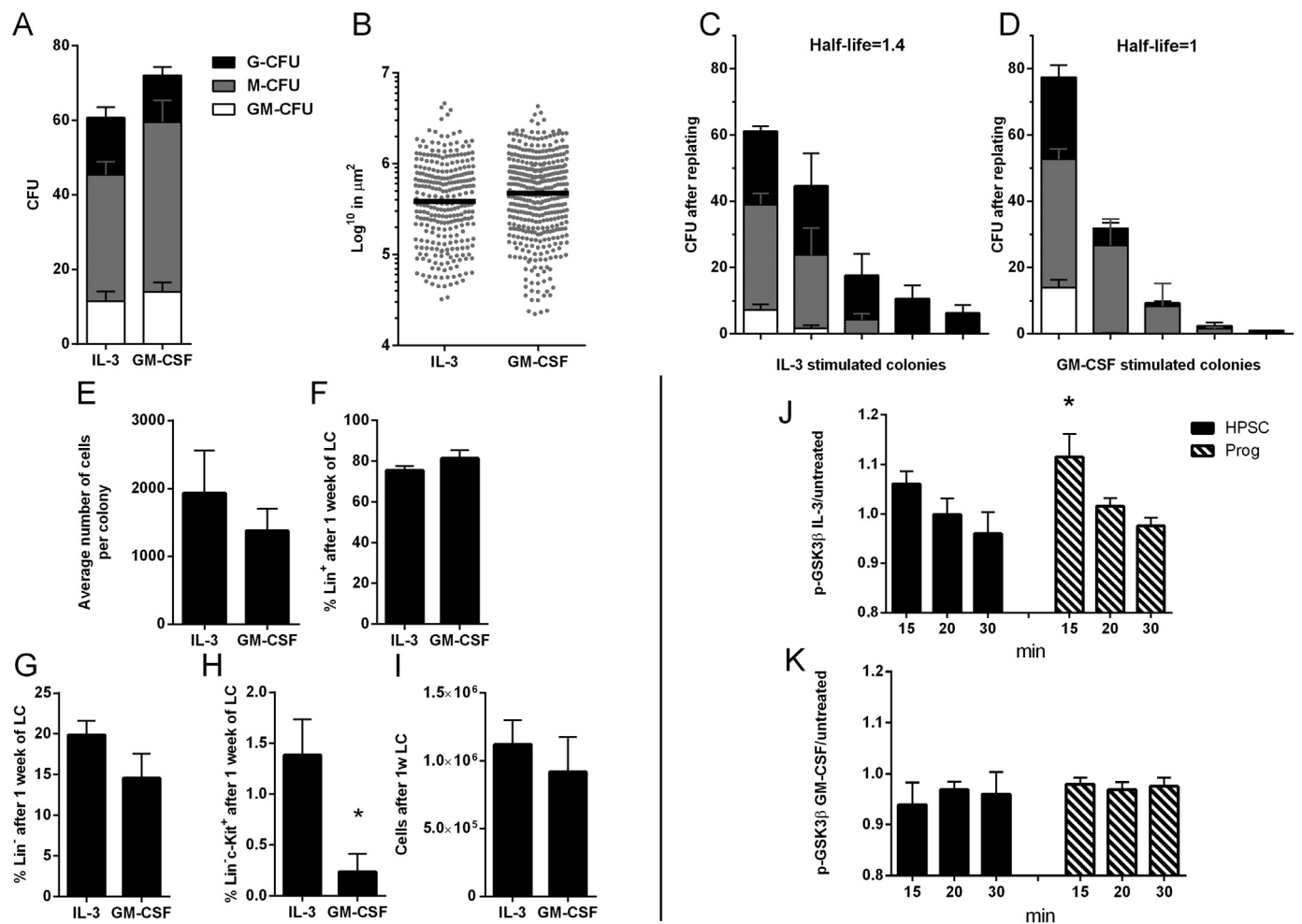
Fig. 3. *Similar effects of IL-3 and GM-CSF on myeloid differentiation.* A) CFU assay after treatment with IL-3 (10 ng/mL) and GM-CSF (10 ng/mL). Colors represent different types of myeloid colonies – GM-CFU = Granulocyte-monocyte colony formation unit (white); M-CFU = Monocyte colony formation unit (grey); G-CFU = Granulocyte colony formation unit (black). B) Log^{10} transformed colony-areas in μm^2. C) CFU replating assay after treatment with IL-3. Colors represent the same types of colonies as described for panel A. Half-life represents calculation of the number of rounds needed to reach a 50% decay in colony formation. D) CFU replating assay after treatment with GM-CSF. Colors represent the same types of colonies as described for panel A. Half-life represents calculation of the number of rounds needed to reach a 50% decay in colony formation. E) Cells/colony after IL-3 and GM-CSF treatment. F) Cell number after 1 week of LC with IL-3 and GM-CSF treatment. G) Percentage of Lin^+ cells after 1 week of liquid culture (LC) with IL-3 and GM-CSF treatment. H) Percentage of Lin^+ cells after 1 week of liquid culture (LC) with IL-3 and GM-CSF treatment. I) Percentage of Lin^-c-Kit^+ cells after 1 week of liquid culture (LC) with IL-3 and GM-CSF treatment. J-K) Analysis of phosphorylation of GSK3β after 15, 20 and 30 min of stimulation with IL-3 or GM-CSF (respectively) in HSPC (Lin^- Sca-1$^+$c-Kit$^+$ − black bars) and more committed progenitors (Lin^-Sca-1$^-$c-Kit$^+$ − stripped bars). Comparison was done between HPSC and progenitors in each treatment. Data are presented as mean ± SEM, except for area data, which is plotted as individual colonies and their median. *$p < .05$.

(this data is highlighted in Supplementary Fig. 3B, where it is also easy to compare with the lack of Wnt5b influence in presence of IL-3). Wnt5b decreased gene expression of Dkk1 and tended to reduce Axin2 expression in the second week of CFU (0.01 ± 0.0 for GM-CSF and 0.00 ± 0.0 for GM-CSF + Wnt5b, p < .05, Figs. 5K and L). Genes involved in non-canonical Wnt signaling (c-Fos, c-Jun and Cdc42) were also analyzed by RT-PCR and, opposite to what was observed for IL-3, in the presence of GM-CSF, Wnt5b upregulated these noncanonical pathway genes.

Interestingly, at the same time we observe an increase in Lin$^+$ population with Wnt5b treatment in presence of GM-CSF, we also observe early apoptosis in the Lin$^-$. Both events might be involved in the functional effects observed.

4. Discussion

In the hematopoietic system, Wnt ligands appear to have roles in lineage specification, differentiation control and cell potential [8,34,42,48,58]. Mounting evidence suggests that increased Wnt5

ligand subfamily expression in the ageing bone marrow microenvironment may drive age-related increases in myeloid differentiation [19]. To understand the imbalances in the ageing environment, first we needed to investigate the roles of Wnt5 ligands in normal physiology in more detail. We thus investigated the roles of Wnt5a and Wnt5b in IL-3- and GM-CSF-induced HSPC myeloid differentiation using a methylcellulose CFU assay. We report that Wnt5a had surprisingly little effect on myeloid differentiation in combination with either IL-3 or GM-CSF. In contrast, Wnt5b had striking, yet divergent effects on myeloid differentiation in combination with IL-3 and GM-CSF. Divergent effects between Wnt5a and Wnt5b have been described in other tissues as well [72]. However, since Wnt5b has not been studied as well as Wnt5a, very little is known on its role in the hematopoietic system. While IL-3-mediated myeloid differentiation was largely repressed by Wnt5b, GM-CSF-induced myeloid differentiation was augmented by Wnt5b. Furthermore, in the presence of IL-3, Wnt5b enhanced primitive cell self-renewal, whereas in the presence of GM-CSF, Wnt5b accelerated differentiation leading to progenitor cell exhaustion. Our results highlight discrepancies between IL-3 and GM-CSF, and reveal novel effects of
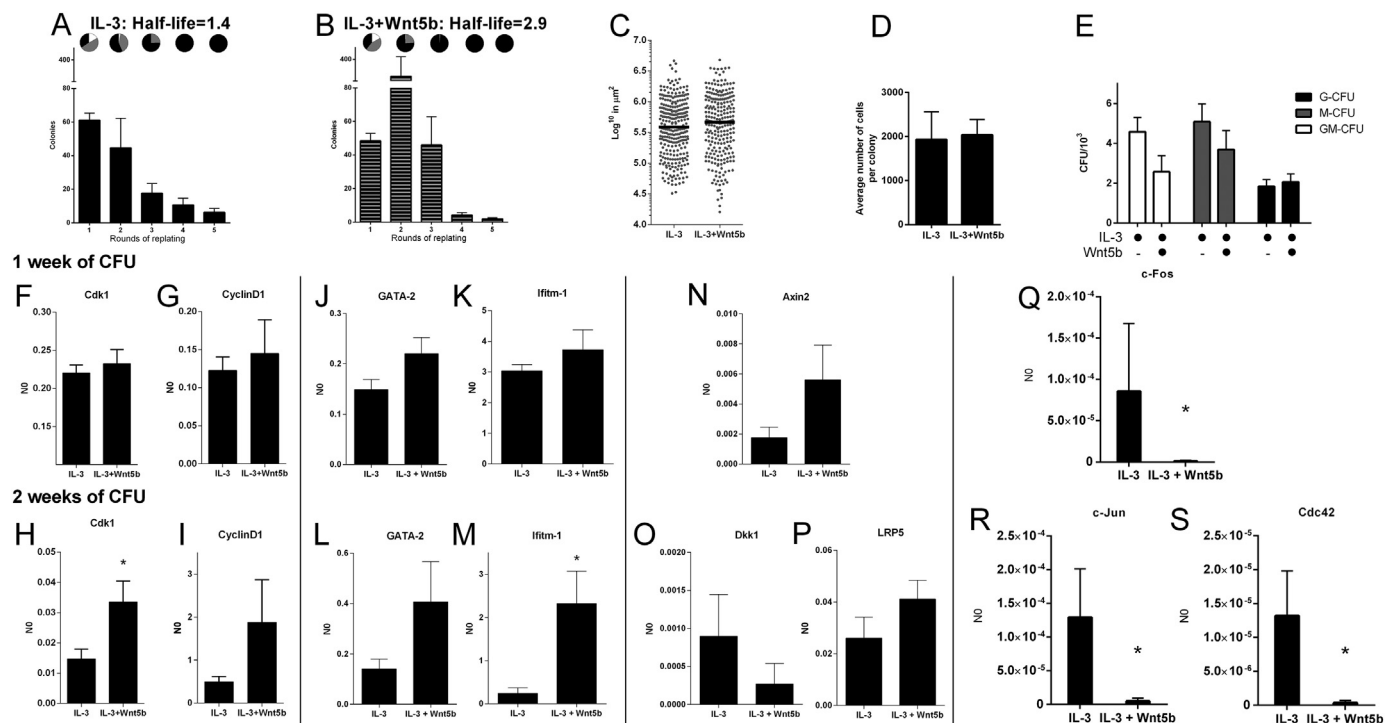
Fig. 4. Wnt5b maintains primitive progenitors in combination with IL-3. A) CFU Replating assay in the presence of IL-3 (10 ng/mL). B) CFU Replating assay in the presence of Wnt5b (200 ng/mL) and IL-3. Bars show total colony number; pie charts represent types of colonies in each round of CFU reading, being white = GM-CFU; grey = M-CFU and black = G-CFU. C) Colony-areas. D) Cell number/colony after IL-3 and IL-3 + Wnt5b. E) CFU replating of subtypes of colonies after IL-3 and IL-3 + Wnt5b. Colors represent GM-CFU = white, M-CFU = grey; G-CFU = black. F–I) Cdk1 (F,H) and Cyclin D1 (G,I) gene expression in relation to housekeeping genes after 1 week (F,G) and 2 weeks (H,I) of CFU assay. J-M) GATA-2 (J,L) and Ifitm-1 (K,M) gene expression in relation to housekeeping genes after 1 week (J,K) and 2 weeks (L,M) of CFU assay. N–P) Axin2 (N), Dkk1 (O) and LRP5 (P) gene expression in relation to housekeeping genes after 2 weeks of CFU assay. P) LRP5 gene expression in relation to housekeeping genes after 2 weeks of CFU assay. Q-S) Gene expression of proteins involved in non-canonical Wnt signaling (c-Fos, c-Jun and Cdc42) in relation to housekeeping genes after 2 weeks of CFU assay. For gene expression, housekeeping genes used were GAPDH and β-actin. Data are presented as mean ± SEM. *p < .05.

Wnt5b on the hematopoietic system.

The divergent outcomes for Wnt5a and Wnt5b treatment we observed are of interest, as these ligands are highly structurally related and share significant amino acid similarity (83%) [31]. Published data on the role of Wnt5 ligand subfamily signaling in hematopoietic differentiation to date relates to the role of Wnt5a, whereas there are no reports about Wnt5b regulation of cytokine-driven granulocyte and monocyte differentiation thus far. The limited knowledge available on the role of Wnt5b in hematopoiesis is restricted to lymphocyte and megakaryocyte differentiation [5,39,41]. Wnt5b does appear to promote thrombopoiesis via G-protein and Ca^{2+} signaling in a zebrafish model [39]. Notably, we observed that Wnt5b had divergent effects depending on the cytokine used to support myeloid maintenance and development.

The maintenance of progenitor cell phenotype by Wnt5b treatment in presence of IL-3 might explain why Wnt5b caused an initial decrease in the number of colonies in the CFU assay, followed by an increase in the 2nd round, as primitive cells cycle less [1,7,11], forming fewer colonies than differentiating cells in the CFU assay [46]. Our data suggest inhibition of IL-3-induced differentiation by Wnt5b. In support of this idea, expression of the cell cycle markers Cdk1 and Cyclin D1, and of the primitive cell markers GATA-2 and Ifitm-1, were increased by Wnt5b in the presence of IL-3. Thus, on a background of IL-3 activation, Wnt5b may stimulate multipotent progenitor cell self-renewal. There is no description of Wnt5b effects on cytokine-driven myelopoiesis whatsoever in literature, so it is the first time it is described opposite results between IL-3 and GM-CSF in presence of Wnt ligands.

In contrast, we observed that in the presence of GM-CSF, Wnt5b caused an increase in colony formation in the first round of the CFU assay, whereas this capability decreased in later rounds. Therefore, on a background of GM-CSF activation, which promotes proliferation [49], Wnt5b induced differentiation of committed progenitors and consequent loss of progenitor cell potential. Furthermore, some of the effects of Wnt5b were observed at the lower concentration in combination with GM-CSF already, whereas in combination with IL-3 only the highest concentration had effects. A possible explanation for this discrepancy is that Wnt5b can target multiple receptors, with a specific receptor population being activated at the 50 ng/mL concentration that regulates GM-CSF responses but not IL-3 responses.

The observed differences are quite striking, as IL-3 and GM-CSF have broadly similar effects on myeloid differentiation [2,9,44,47]. The receptors for IL-3, GM-CSF and IL-5 share a common β-chain [22] which via interaction with their ligands can stimulate intracellular signaling pathways such as JAK/STAT, Ras/ERK, PI3K/PKB, PLCγ2 and PKCβ/RACK1 [22,35]. Wnt signaling has been linked to the activation of Ras/ERK, PI3K/PKB, PLCγ2 and PKCβ/RACK1 [10,13,28,33,59,69] and these all represent potential points of convergence in the signaling interactions between Wnt5b and IL-3/GM-CSF.

Another explanation for the divergent effects of Wnt5b after IL-3 or GM-CSF stimulation could be related to differences in expression of the IL-3 and GM-CSF receptor α-chains between different cell populations present in the assay [68]. Although IL-3 and GM-CSF produced similar numbers of colonies of comparable sizes, IL-3 treatment generated more primitive (Lin⁻Sca1⁺) cells than GM-CSF. Notably, the IL-3 receptor α-chain was described to be enriched in myeloid progenitor cells relative to more differentiated hematopoietic cell types [43]. In contrast, the GM-CSF receptor α-chain was largely not expressed by primitive, colony-forming bone marrow cells, whereas higher expression was observed in more differentiated monocyte precursors, granulocytes and monocytes. Thus, due to differential cognate receptor expression, IL-3
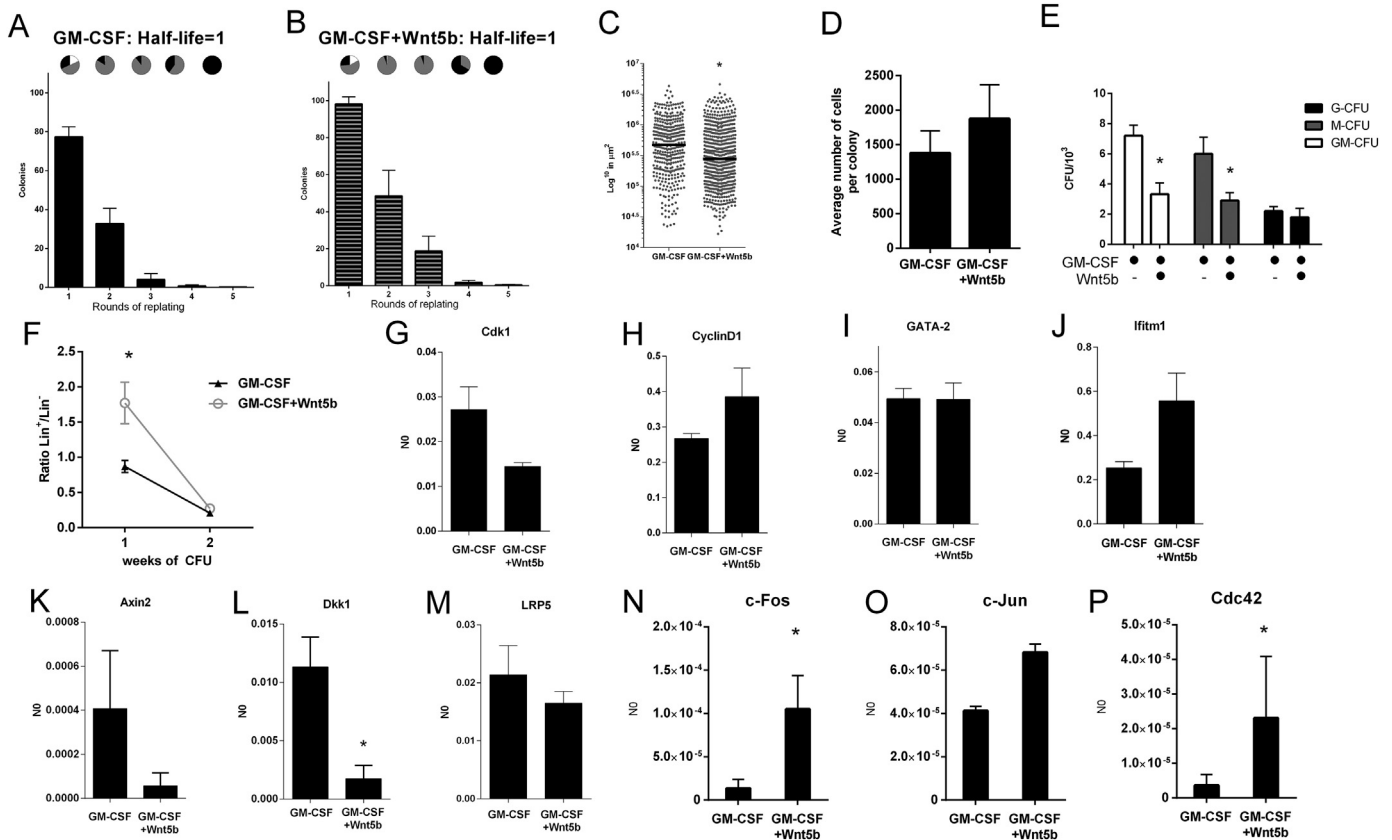
Fig. 5. Wnt5b induces committed progenitor activation and progenitor exhaustion in the presence of GM-CSF. A) CFU Replating assay in the presence of GM-CSF (10 ng/mL). B) CFU Replating assay in the presence of Wnt5b (200 ng/mL) and GM-CSF. Pie charts represent types of colonies in each rounds of CFU reading, being white = GM-CFU; grey = M-CFU and black = G-CFU. C) Colony-areas. D) Cell number/colony after GM-CSF and GM-CSF + Wnt5b treatment. E) CFU replating of subtypes of colonies after GM-CSF and GM-CSF + Wnt5b treatment. F) Ratio between Lin + and Lin- populations after 1 and 2 weeks of CFU assay. G-L) Cdk1 (G), Cyclin D1 (H), GATA-2 (I), Ifitm-1 (J), Axin2 (K), Dkk1 (L), LRP5 (M), c-Fos (N), c-Jun (O) and Cdc42 (P) gene expression in relation to housekeeping genes after 2 weeks of CFU assay. For gene expression, housekeeping genes used were GAPDH and β-actin. Data are presented as mean ± SEM. *$p < .05$.

could enrich for progenitor cells, whereas GM-CSF could enrich for differentiated myeloid cell types; these populations could in turn exhibit divergent responses to Wnt5b. The viability data support this and indicate that Lin$^-$ cells are better maintained than the Lin$^+$ cells by Wnt5b in combination with IL-3, whereas for GM-CSF this exactly the other way around. Another possibility is that Wnt5b is inducing cytokine expression by the hematopoietic cells, as already described in lung fibroblasts [67], with divergent downstream effects on IL-3 and GM-CSF signaling. The latter is more likely, as we analyzed whether Wnt5b could interfere in the expression of IL-3 and GM-CSF receptors and no changes were observed, either in alpha or beta chains.

The finding that IL-3 increased the number of primitive cells compared to GM-CSF may be related to stimulation of Wnt/β-catenin signaling, as IL-3 triggered phosphorylation of GSK3β, and IL-3-treated colonies had higher baseline gene expression of Axin2, a canonical Wnt/β-catenin target gene, compared to GM-CSF-treated colonies. Notably, Wnt5b augmented Axin2 gene expression in the presence of IL-3, yet decreased Axin2 expression in the presence of GM-CSF. Thus, primitive hematopoietic cells may be primed to activate Wnt/β-catenin signaling in response to Wn5b. In support of this, Wnt5 signaling activated Wnt/β-catenin signaling in short-term HSCs, yet repressed Wnt/β-catenin signaling in more differentiated progenitors [65,67]. Accordingly, we also found that a Wnt/β-catenin pathway agonist (CHIR99021) had opposite effects to Wnt5b when combined with GM-CSF (Supplementary Fig. 4). However, in spite of these data, we found no obvious GFP signal in bone marrow-derived cells of TCF/Lef:H2B-GFP fusion Wnt reporter mice [18] and could not detect overt localization of β-catenin protein using immunofluorescence microscopy,

either at baseline or in response to cytokine stimulation. These results are reinforced by western-blotting results, which show no difference between cells treated with IL-3, IL-3 + Wnt5b, GM-CSF or GM-CSF + Wnt5b, for β-catenin expression (data not shown). Thus, whereas regulation of Wnt/β-catenin signaling by Wnt5b represents one possible explanation, it is likely that alternative signaling pathways participate in its effects. Such mechanisms may include typical noncanonical Wnt pathway activation signals such as Rho/Cdc42/Rac pathways. These pathways were previously reported to contribute to cell signaling in ageing hematopoietic stem cells in response to Wnt5a [19] and clearly this needs to be investigated further in future studies.

Our findings show no major effects of Wnt5a on IL-3 and GM-CSF-driven myelopoiesis, in contrast to the significant and divergent effects of Wnt5b. Despite 83% sequence homology [31], Wnt5a and Wnt5b have non-overlapping effects in other cell systems including lung fibroblasts [61,67] and alveolar epithelial progenitor cells of the lung (Wu, X. et al., unpublished observations). Unfortunately, limited knowledge on FZD receptor subtype selectivity of Wnt5b is available. Nonetheless, divergent FZD receptor subtype binding and activation by these two ligands is the most likely explanation for the observed functional differences between Wnt5a and Wnt5b.

It is too early to propose therapeutic uses of Wnt5a or Wnt5b antagonists against ageing-associated myeloid imbalances, even more considering the link between distorted Wnt signaling and leukemia development [23,27,40,62,70]. However, the accumulating evidence that switching from canonical to non-canonical Wnt signaling regulates key cellular features of hematopoietic ageing and chronic disease [19,67], warrants further investigation into the therapeutic

opportunities associated with inhibition of non-canonical Wnt signaling. In this context, our results highlight important discrepancies between Wnt5a and Wnt5b, and show novel effects of Wnt5b on the hematopoietic system.

Declaration of Competing Interest

The authors declare no competing interests, financial or not financial.

Acknowledgements

Author contributions

MMdR participated on experimental work and data collection, MMdR, JPNB and RG participated on research design and data analysis. All the results discussion data interpretation, literature overview and manuscript preparation was performed by all authors – MMdR, JPNB, GZJ, EJPG and RG.

Data availability statement

All data is available, as well as the methods used for collecting the data. There is no restriction on the availability of data or information.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2019.109507.

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