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Grb2 regulates the proliferation of hematopoietic stem and progenitors cells

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ABSTRACT

Although Hematopoietic Stem and Progenitor Cell (HSPC) proliferation, survival and expansion have been shown to be supported by the cooperative action of different cytokines, little is known about the intracellular signaling pathways that are activated by cytokines upon binding to their receptors. Our study showed that Growth factor receptor-bound protein 2 (*Grb2*) mRNAs are preferentially expressed in HSC compared to progenitors and differentiated cells of the myeloid and erythroid lineages. Conditional deletion of *Grb2* induced a rapid decline of erythroid and myeloid progenitors and a progressive decline of HSC numbers in steady state conditions. We showed that when transplanted, *Grb2* deleted bone marrow cells could not reconstitute irradiated recipients. Strinkingly, *Grb2* deletion did not modify HSPC quiescence, but impaired LT-HSC and progenitors ability to respond a proliferative signal induced by 5FU *in vivo* and by various cytokines *in vitro*. We showed finally that *Grb2* links IL3 signaling to the ERK/MAPK proliferative pathway and that both SH2 and SH3 domains of *Grb2* are crucial for IL3 signaling in progenitor cells. Our findings position *Grb2* as a key adaptor that integrates various cytokines response in cycling HSPC.

1. Introduction

Hematopoietic stem and progenitor cells (HSPC) represent a population of cells that have the unique capacity to both produce large pools of self-sustaining cells and to differentiate into all blood lineages upon transplantation into irradiated hosts. Despite the fact that these properties have been successfully exploited for clinical transplants for more than four decades, the intracellular signaling pathways that regulate HSPC proliferation and expansion are not characterized. HSPC are characterized by different subtypes. HSC have the ability to self renew and sustain multi-lineage engraftment. But while Long-Term (LT)-HSC have the ability to do so indefinitely, Intermediate-Term HSC (IT-HSC) and Short-Term HSC (ST-HSC) loose this ability over time. Committed progenitors only induce a rapid and transient production of blood cells when transplanted into irradiated recipients. LT-HSC can be purified from mouse bone marrow to near homogeneity and separated from the transiently engrafting Intermediate-Term HSC (IT-HSC) using CD150 and CD49b cell surface markers [1–4]. LT-HSC are deeply quiescent with very rare mitotic intervals (every 50–100 days) during homeostasis [5,6] but can be induced to cycle by inflammatory signals [7–11], myelo-ablative treatment [12,13] and have been shown to cycle and expand upon serial transplantations into irradiated hosts [14,15]. Many efforts have been made to identify extrinsic regulators secreted by the irradiated environment that induce HSPC expansion upon transplantation that could be used for *ex vivo* expansion strategies. Studies have showed that Hematopoietic Stem and Progenitor Cell (HSPC) proliferation, survival and to a lesser extent expansion can be supported *in vitro* by various cytokines, including Fl3-ligand, Stem Cell Factor (SCF), interleukin-11 (IL-11), interleukin-3 (IL-3) and thrombopoietin (TPO) alone or in synergy with other cytokines [16–21]. In *in vivo* LT-HSC expansion can be enhanced by the injection of SCF and IL-11 after transplantation into irradiated recipients [15]. However,

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despite the above, little is known about the intracellular signaling activated by the binding of cytokines to their receptors in proliferative HSPC.

Cytokines bind specific membrane receptors endowed with tyrosine kinase activity. Cytokine binding triggers receptor phosphorylation on tyrosine residues within specific structure motifs which induces the binding of signaling molecules such as SH2 domain containing proteins. Among them, the growth factor receptor-binding protein 2 (*Grb2*) contains a single SH2 domain, flanked by two SH3 domain, thus having the capacity to link activated receptor tyrosine kinase (RTK) to various targets such as the SOS guanine nucleotide exchange factor (GEF) which promotes the activation of the Ras/MAPK pathway. *Grb2* has also been shown to interact with scaffold proteins SHC-1 (definer), GAB1, GAB2, and the phosphatase SHP-2 which are downstream effectors of IL-11, IL-3 and SCF cytokine induced signaling [22]. Although *Grb2* was shown to link the signaling induced by Flt3, IL-3, SCF and GM-CSF to the Ras/MAPK (mitogen-activated protein kinase) signaling pathway and to control cell proliferation and survival in various normal and cancerous hematopoietic cell lines [23–25], the role of *Grb2* in cytokine-induced proliferation and survival of adult HSPC has not been explored.

Grb2 is required for primitive endoderm (PE) differentiation, placental development and survival of migrated neural crest cells [26–28]. As a consequence, embryos deficient for *Grb2* die at embryonic day (E) 4.5 [29]. We used a conditional mouse model [30] to show that *Grb2* deletion didn't affect mice life span but induced a rapid decline of progenitor and mature cells from the myeloid, erythroid and lymphoid lineages and a progressive decline of LT and IT-HSC. *Grb2* deletion impaired the ability of bone marrow cells to reconstitute hematopoiesis when transplanted into irradiated recipients. *In vivo Grb2* deleted Hematopoietic Stem and Progenitor Cells (HSPC) were normally quiescent but their ability to exit quiescence and to proliferate in response to a 5-FU proliferative signal was impaired. *In vitro*, *Grb2* deleted LT-HSC could exit quiescence but could not cycle more than once or twice when cultured with serum and cytokines. Moreover, *Grb2* deleted progenitors could not proliferate and differentiate into colonies from the erythroid and myeloid lineages. We showed finally that *Grb2* links IL3 signaling to the ERK/MAPK proliferative pathway and that both SH2 and SH3 domains of *Grb2* are crucial for IL3 signaling in progenitor cells.

2. Results

2.1. Grb2 mRNAs expression in the hematopoietic hierarchy

To examine the role of *Grb2* in HSPC, we first analyzed *Grb2* mRNA expression in HSC and in progenitors and mature cells of the hematopoietic hierarchy. We found that *Grb2* transcripts were preferentially expressed in HSC compared to other cells from the hematopoietic hierarchy (Fig. 1). When we examined *Grb2* mRNA expression in the different HSC subtypes we found that *Grb2* transcripts levels were not significantly different between LT-HSC, IT-HSC and ST-HSC, however they were 2 to 4 times higher in HSC, compared to progenitor cells of the erythroid and myeloid lineages and to some mature cells (erythrocytes, neutrophils and megakaryocytes) (Fig. 1A). We also examined *Grb2* mRNA expression in different fractions of immature and mature B and T lymphoid cells (Fig. 1B and C) and found that *Grb2* mRNAs were 4 to 50 times and 2 to 7 times higher in the different fractions of B and T cells tested compared to LT-HSC.

All together this mRNA expression analysis supports the described role of *Grb2* in B and T cells differentiation [31,32] [33] and suggests a physiological role for *Grb2* in HSC and progenitor cells of the hematopoietic hierarchy.

2.2. Effect of Grb2 deletion on steady state hematopoiesis

To test the physiological role of *Grb2* in hematopoiesis, we used a conditional $Grb2^{fl/fl}$ KO mouse model [34] that we bred to Mx1-Cre mice to obtain conditional deletion of *Grb2* in hematopoietic cells by injection of polyinosinic-polycytidylic acid (poly(I:C)). All control ($Grb2^{fl/fl}$ = WT) and mutant ($Grb2^{fl/fl}$-Mx1-Cre = KO *Grb2*) mice were treated with four injections of poly (I:C). We found that mutant mice were healthy and their longevity was normal despite a significant decrease of *Grb2* protein amounts as shown by western blot analysis of total bone marrow cells 14 days after the last poly(I:C) injection (Fig. 2A). A rapid blood cell analysis revealed however that white and red blood cells numbers were substantially decreased in mutant mice one month after *Grb2* deletion by poly(I:C) (Fig. 2B). A closer look at WT and KO *Grb2* bone marrows revealed that one month after *Grb2* deletion, total bone marrow cells numbers were 2-fold lower in mutant mice compared to controls and that common myeloid progenitors (CMP), myeloid/erythroid progenitors (MEP) and granulocyte/macrophage progenitors (GMP) numbers were 5-fold lower in mutant mice compared to controls (Fig. 2C).

Because the reduction of progenitor cell numbers could be explained by the shrinkage of the HSC pool in mutant marrows, we next analyzed the numbers of $Lin^-Sca1^+cKit^+$ (LSK), $Rho^{lo}Lin^-Sca1^+cKit^+CD150^-CD49b^+$ ($RLSKCD150^-CD49b^+$ = IT-HSC) and $Rho^{lo}Lin^-Sca1^+cKit^+CD150^+CD49b^-$ ($RLSKCD150^+CD49b^-$ = LT-HSC) in control and mutant mice 1 month after *Grb2* deletion by poly(I:C). We found that although LSK numbers were 4 fold lower in mutant mice compared to controls, LT-HSC and IT-HSC numbers were similar (Fig. 2D). We next analyzed LT-HSC and IT-HSC numbers and frequencies in mice 8 months after *Grb2* deletion and found that although LT-HSC and IT-HSC frequencies were unchanged, both LT-HSC and IT-HSC numbers were 5 fold lower in mutant mice compared to controls (Fig. 2E and Supplementary Fig. 1).

All together, these results show that *Grb2* deletion in hematopoietic cells induces a rapid depletion of progenitor cells and a progressive depletion of HSC without affecting mice survival.

2.3. Effect of Grb2 deletion on HSPC bone marrow reconstitution

To determine whether *Grb2* deletion impairs HSC function, we performed a competitive repopulation assay in which 5×10^6 donor KO *Grb2* or WT unfractionated bone marrow cells were collected and injected into lethally irradiated recipient mice in competition with 10^6 unfractionated bone marrow recipient cells. We next measured donor-derived lineage reconstitution in blood 8, 16 and 24 weeks after the transplant. This experiment showed that KO *Grb2* bone marrow cells failed to regenerate short-term and long-term engraftment of any of the lymphoid, myeloid or erythroid lineages (Supplementary Fig. 2A) when injected in competition with WT bone marrow cells.

In order to determine if KO *Grb2* bone marrow regenerative defect was due to an inability of KO *Grb2* cells to home into recipient marrows, we transplanted 10^7 WT or KO *Grb2* unfractionated bone marrow cells previously labeled with the CMFDA CellTracker fluorescent dye into lethally irradiated recipients and analyzed the percentage of fluorescent LSK cells in recipient marrows 24 h later. We found that the percentages of fluorescent LSK cells in recipient marrows were similar in mice transplanted with WT or KO *Grb2* bone marrow cells. This suggests that KO *Gbr2* HSPC home with the same efficiency than WT cells into irradiated recipients (Supplementary Fig. 2B).

In order to determine if the regenerative defect induced by KO *Grb2* bone marrow cells was cell autonomous or if it was a consequence of *Grb2* systemic deletion in poly(I:C) treated mice, we transplanted 10^6 unfractionated donor bone marrow cells, collected from $Grb2^{fl/fl}$ (WT) or $Grb2^{fl/fl}$-Mx1Cre (KO *Grb2*) mice in competition with 10^6 recipient bone marrow cells, into lethally irradiated recipient mice. We first analyzed the donor-derived multi-lineage reconstitution in blood 8 weeks after bone marrow transplantation before inducing *Grb2* deletion with 4 injections of poly(I:C). We next analyzed donor-derived multi-lineage reconstitution in blood 4, 8, 16 and 24 weeks after *Grb2* deletion by poly(I:C) (12, 16, 24 and 32 weeks post-transplant). We