Micro-environmental sensing by bone marrow stroma identifies IL-6 and TGFβ1 as regulators of hematopoietic ageing

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Hematopoietic ageing involves declining erythropoiesis and lymphopoiesis, leading to frequent anaemia and decreased adaptive immunity. How intrinsic changes to the hematopoietic stem cells (HSCs), an altered microenvironment and systemic factors contribute to this process is not fully understood. Here we use bone marrow stromal cells as sensors of age-associated changes to the bone marrow microenvironment, and observe up-regulation of IL-6 and TGFβ signalling-induced gene expression in aged bone marrow stroma. Inhibition of TGFβ signalling leads to reversal of age-associated HSC platelet lineage bias, increased generation of lymphoid progenitors and rebalanced HSC lineage output in transplantation assays. In contrast, decreased erythropoiesis is not an intrinsic property of aged HSCs, but associated with decreased levels and functionality of erythroid progenitor populations, defects ameliorated by TGFβ-receptor and IL-6 inhibition, respectively. These results show that both HSC-intrinsic and -extrinsic mechanisms are involved in age-associated hematopoietic decline, and identify therapeutic targets that promote their reversal.

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During ageing erythropoiesis and lymphopoiesis both decrease. The former leads to a significantly increased frequency of anaemia in the elderly population, which reaches 25–30% at 85 years of age. Anaemia is clinically defined as low blood haemoglobin (Hb) (≤12 g/dl in women, ≤13 g/dl for men), and is associated with several key age-related morbidities, including cognitive, musculoskeletal and cardiovascular decline, as well as higher overall mortality. Decreased lymphopoiesis impairs de novo generation of naïve B-cells and T-cells. This leads to a declining antibody- and T-cell receptor repertoire and impaired immune response to key pathogens such as respiratory syncytial virus and influenza and their vaccines. Understanding the underlying causes of the age-associated decrease in erythropoiesis and lymphopoiesis, and the development of pharmacological strategies to counteract them, is therefore an important step towards improving the health of the ageing population.

Hematopoietic stem cells (HSCs) maintain the hematopoietic system throughout the lifespan of the vertebrate organism. However, the HSC compartment, as well as the properties of individual HSCs, undergoes significant changes with age. Repopulating HSCs expand7,8, while their individual ability to generate differentiated progeny decline9,10. In addition, HSCs are heterogeneous in their lineage output, as demonstrated by single HSC transplantation11–14, and the proportions of fate-restricted and lineage-biased HSCs change with age: HSCs with platelet and myeloid lineage bias become more prevalent, while the frequency of HSCs that produce lymphoid cell types decreases15–17. The latter observation provides a cellular basis for the age-dependent decline in de novo production of naive B- and T-lymphocytes18.

While age-dependent changes to HSC function and lineage output are well established, the underlying causes are less well understood. The functional decline of HSCs has been linked to their proliferative history, as highly quiescent HSCs retain their repopulating capacity over time19,20. Increased molecular and functional platelet bias of aged HSCs has been observed in both mice and humans17,21, and HSC platelet programming may directly inhibit lymphocyte production17. In contrast, we do not know how ageing affects the intrinsic ability of HSCs to produce erythroid cells. This is principally due to technical limitations: identification of the cellular output from HSCs has traditionally used CD45 allotypic markers, and erythrocytes do not express CD45. Therefore, quantitative studies measuring functional differences between young and aged HSCs have thus far not addressed their intrinsic capacity for erythropoiesis or altered.

Age-dependent intrinsic changes to HSC lineage output may be influenced by extrinsic factors: Ccl522 and Wnt5a23 have both been observed to promote age-dependent myeloid bias, as has the age-dependent loss of β-adrenergic innervations in the bone marrow24, with the caveat that these studies did not measure platelet contributions from HSCs. Finally, the circulating levels of inflammatory cytokines, such as IL-6 and TNFα, increase during normal ageing in both mice25 and humans26, with the levels both cytokines similarly increased in bone marrow27,28 and have been shown to inhibit erythropoiesis both in vitro28 and in vivo29. However, their impact on HSC and downstream progenitor function during physiological ageing remains to be experimentally determined.

We here use comprehensive readout of hematopoietic lineage in competitive repopulation assays to show that reduced lymphoid, but not erythroid, lineage output is an intrinsic property of aged HSCs. By gene expression profiling of multiple stromal populations from young and aged bone marrow and pathway analysis we identify TGFβ and IL-6 signalling as increased in the aged micro-environment. Inhibition of IL-6 signalling in aged mice using a neutralising anti-IL-6 antibody reverted the age-associated decrease in erythroid progenitor activity. Inhibition of TGFβ signalling rebalanced the lineage output from aged repopulating HSCs and increased their lymphoid progenitor production, and in addition ameliorated the decrease in erythroid progenitor numbers seen in aged mice. Therefore, inhibition of IL-6 and TGFβ signalling in aged mice co-operatively counteract the age-associated decrease in erythroid and lymphoid cell production.

Results

Decreased erythroid output is not intrinsic to aged HSCs. As observed during human ageing, aged mice have lower haematocrit (HCT) and Hb levels compared to young mice, whereas their platelet count is increased (Fig. 1a). To determine whether decreased erythropoiesis is due to an intrinsic defect of aged HSCs we transplanted phenotypic long-term (LT-)HSCs (Lin- c-Kit+ Sca-1+ CD150+ CD48- CD34+)30,31 (Fig. 1b) from young (2–3-month-old) and aged mice (24-month-old) carrying the Vwf-tdTmato and Gata1-EGFP transgenes (VT/GE mice), which allow for accurate readout of erythrocytes, which are labelled by EGF+ and as platelets, which are both EGF+ and tdTomato+14. Fifty VT/GE long-term HSCs (CD45.2 allele) were transplanted into young CD45.1 recipients along with 200,000 young wild-type CD45.1 competitor cells (Fig. 1c) and long-term reconstitution of all hematopoietic lineages readout using CD45 allotypes (Fig. 1d) and the Vwf-tdTmato and Gata1-EGFP transgenes (Fig. 1e, f). As previously reported, aged HSCs show an overall lower level of lineage reconstitution, with a particularly prominent decrease in lymphocyte output11,29,30,31 while for platelets, erythrocytes and myeloid cells a 1.5–2-fold decrease in reconstitution was seen, this difference was fourfold to fivefold for B-cells and T-cells (Fig. 1g). Since the overall number of repopulating HSCs increases with age, compensating for the decrease in lineage output on a per cell basis, the critical issue is their relative contribution to hematopoietic lineages. Normalisation to platelet output, which is the lineage with the highest output from both young and aged HSCs, as well as single fate-restricted HSCs14, showed that lymphoid output from aged HSCs was decreased, whereas myeloid and erythroid-lineage output did not decline relative to platelet output during ageing (Fig. 1h). Decreased erythroid-lineage output is therefore not an intrinsic property of aged HSCs, whereas decreased lymphoid cell output is.

The function of erythroid progenitors decrease with age. To address whether specification or differentiation of erythroid-lineage progenitors was impaired in an aged bone marrow environment, we compared the number and function of hematopoietic stem and progenitor cells between young and aged mice. As previously reported, during ageing the frequency of LT-HSCs and multi-potent (LSK) progenitor cells increases (Fig. 2a, b). We also observed an increase in total Lin- c-Kit+ (LK) myelo-erythroid progenitors (Fig. 2c). Analysis of the LK compartment12 (Fig. 2d) showed an increase in megakaryocyte (MkP) and myeloid (GM) progenitors, whereas the frequency of erythroid-lineage progenitors (preMegE, preCFU-E, CFU-E) (Fig. 2e) was significantly decreased. To measure the functional state of erythroid progenitors we purified MPP2s, preCFU-Es and CD71+ CFU-Es from young and aged mice and measured their colony-forming capacity, observing a decrease in clonogenic erythroid progenitors for all three populations during ageing (Fig. 2f). Gene expression profiling and gene set enrichment analysis (GSEA) of preCFU-Es showed that expression of genes normally associated with erythroid-lineage specification was significantly decreased in old compared to young mice (Fig. 2g), with a
corresponding increase in preGM-associated gene expression (Fig. 2h). Accordingly, we observed that the aged preCFU-E and MPP2 populations, in addition to their lower erythroid output, also displayed a significant increase in myeloid colony formation (Fig. 2i).

Identification of deregulated signalling in aged bone marrow. The bone marrow micro-environment is under the combined influence of locally and systemically produced factors. To assess the overall effect of ageing on the bone marrow micro-environment at the molecular level, we performed RNA sequencing of sorted bone
Fig. 1 Effect of ageing on erythropoiesis. a PB haemoglobin (Hb), haematocrit (HCT) and platelets number measured in young (red cell parameters: N = 16; platelets: N = 11) and old mice (red cell parameters N = 20; platelets: N = 13). Data are from seven independent experiments. Values show mean ± s.e.m. *P < 0.05; **P < 0.01; ***P < 0.001 (two-tailed, unpaired Student’s t test). Exact P values: Hb: 0.0014; HCT: 0.026; Platelets: 0.000003. b Gating strategy for sorting of LT-SCSs from young and old VT/GE CD45.2 transgenic mice used as donors for competitive transplantation. c Experimental design to compare in vivo lineage output of transplanted young and old LT-SCSs. Strategy for measuring donor PB leucocyte (d), erythrocyte (e) and platelet reconstitution (f) in transplanted recipient. g PB lineage contribution of young and old HSs in transplanted recipients (N = 10/condition). The mean values ± s.e.m are indicated, as are P values (two-sided Mann-Whitney U-test) comparing young and old contributions for each lineage. *P < 0.05; **P < 0.01; ***P < 0.001. Exact P values: PLT: 0.03; ERY: 0.12; MYL: 0.06; BLY: 0.003; TLY: 0.003. h Donor HSC PB contribution from g normalised to platelet output. The mean values ± s.e.m are indicated, as are P values (two-sided Mann-Whitney U-test) comparing young and old contributions for each lineage. *P < 0.05; **P < 0.01. Exact P values: PLT: not applicable; ERY: 0.35; MYL: 0.17; BLY: 0.007; TLY: 0.009). Source data are provided as a source data file.

Fig. 2 IL-6 inhibition improved aged erythroid progenitor function. These observations were consistent with elevated IL-6 signalling in the aged bone marrow environment contributing to the age-dependent decline in erythropoiesis. We therefore treated aged mice with a neutralising anti-IL-6 antibody or control IgG for 3 weeks and characterised their erythroid progenitor compartment both phenotypically and functionally. Treatment with IL-6 antibody did not affect the number of phenotypic HSCs, LSK or LK cells (Supplementary Fig. 3a–c). Analysis of the myeloid-erythroid progenitor compartment showed a small, but significant, increase in phenotypic preMegEs and a similar decrease in GMPs, consistent with IL-6 contributing to myeloid skewing of progenitor differentiation. However, we did not observe any change in the proportion of phenotypic committed erythroid progenitor cells upon IL-6 inhibition (Fig. 3c). To address whether inhibition of IL-6 affected erythroid or myeloid progenitor function we cultured LK cells isolated from anti-IL-6- and control IgG-treated mice under erythroid and myeloid conditions. We observed a highly significant increase in erythroid colony-forming cells (Fig. 3d), and a corresponding decrease in myeloid colony-forming cells (Fig. 3e). Also, the erythroid colony-forming activity of purified preCFU-Es was increased (Fig. 3f). GSEA analysis of anti-IL-6- and IgG-treated preCFU-Es showed that age-associated depletion of preCFU-E-specific gene expression was observed in preCFU-Es from IgG-treated, but not anti-IL-6-treated, mice (Fig. 3g, h), indicating that IL-6 inhibition improves erythroid progenitor function by restoring erythropoiesis-specific gene expression in progenitors. This analysis also showed a lesser degree of upregulation of the preGM signature in preCFU-Es from anti-IL-6-treated aged mice compared to control IgG-treated aged mice; however, this difference was not significant (Supplementary Fig. 3d, e). We did not observe increased expression of Il6 during ageing in any of the stromal cell populations examined (Supplementary Fig. 3f), or change to their expression of key erythroid cytokines (Kitl, Epo; Supplementary Fig. 3g, h). This would be consistent with increased levels of circulating IL-6, rather than altered local production of supporting cytokines in the BM, being responsible for the age-dependent activation of inflammatory signalling pathways in bone marrow stromal cells, and the associated decline in erythropoiesis. To determine the effect of elevated systemic IL-6 levels we overexpressed IL-6 in young mice using hydrodynamic injection, as previously described33. We observed that increased circulating IL-6 (Supplementary Fig. 3i) led to an increase in GMPs and a decrease in preMegEs (Supplementary Fig. 3j), the converse effect of IL-6 inhibition in aged mice, substantiating a direct role of elevated circulating IL-6 levels in the aged progenitor phenotype.

The mean values ± s.e.m are indicated, as are P values (two-sided Mann-Whitney U-test) comparing young and old contributions for each lineage. *P < 0.05; **P < 0.01; ***P < 0.001. Exact P values: PLT: 0.03; ERY: 0.12; MYL: 0.06; BLY: 0.003; TLY: 0.003. Source data are provided as a source data file.

TGFβR1 inhibition reduces HSC number in aged mice. While IL-6 inhibition improved erythroid progenitor function, it did not restore the number of phenotypic erythroid progenitors in aged mice, nor did it counteract the increase in MkPs that is associated with age-dependent HSC platelet bias17. In the Metacore analysis the TGFβ/GDF/activin process network was enriched in 3/6 aged stromal cell populations. Inhibition of TGFβ and activin signalling has been shown to ameliorate ineffective erythropoiesis in myelodysplastic syndrome34 and β-thalassaemia35, respectively. In addition, loss of HSC quiescence was observed after megakaryocyte (MK)-specific ablation of Tgfb136, and MKs are enriched in the niches of Vwf+ platelet-biased HSCs37. Since platelet-biased HSCs are preferentially expanded during ageing37, increased TGFβ signalling therefore had the potential to both impair erythropoiesis and promote HSC platelet bias. We examined the expression of TGFβ family genes encoding between young and aged stromal cell types (Supplementary Fig. 4a). This showed that Tgfb1 expression was strongly and consistently upregulated in aged stromal cells (Supplementary Fig. 4b), while no such upregulation was observed for other known extrinsic regulators of HSC function (Kitl, Cxcl12, Angpt1) (Supplementary Figs. 3g and 4c, d). Consistent with these observations, the level of Tgfb1 protein was elevated in the bone marrow plasma of old compared to young mice (Supplementary Fig. 4e). We therefore treated aged mice with SB-525334 (SB), a selective inhibitor of TGFβ receptor38, the catalytic subunit of the TGFβ receptor59. Examination of HSCs and progenitors after 2 weeks of treatment

linin (BL) osteoblasts (OBs: CD45–Ter119–ALCAM+), mesenchymal stromal cells (MSCs: CD45–Ter119–CD31–ALCAM–Sca-1+; PDGFRα+) and vascular endothelial cells (VECs: CD45–Ter119–CD31+), as well as central bone marrow (CBM) VECs, LepR+PDGFRα+ and LepR+PDGFRα+ perivascular cells (PV–); CD45–Ter119–CD31–LepR+PDGFRα+, PV–; CD45–Ter119–CD31–LepR–PDGFRα–) (Supplementary Fig. 1a, b). The identity of the sorted cell populations was validated by analysing the genes encoding their characteristic markers (Supplementary Fig. 1c). We observed that a large number of genes were regulated by age in all cell types (Fig. 3a and Supplementary Data 1). To identify signalling pathways underlying these gene expression changes we performed Metacore process network analysis, focusing on process networks that were enriched during ageing across multiple aged stromal cell types, as these were likely to reflect general changes to the microenvironment, rather than effects of ageing specific to individual cell types. This analysis revealed that several inflammatory process networks were enriched in the majority of aged BM stromal cell types, most prominently those associated with IL-2, IL-6 and IL-10 signalling (Fig. 3b and Supplementary Data 2). Il1ra was not expressed on stromal cells, and Il1ra at low levels and only in aged OBs and CBM-VECs (Supplementary Fig. 2a, b). In contrast Il6st was expressed at high levels in aged stromal cell types where the IL-6 network was highly enriched, and at low levels in those showing weaker enrichment (Supplementary Fig. 2c). Finally, GSEA showed that IL-6-induced gene expression was significantly enriched in aged mice in the same stromal cell types that showed a high IL-6 process enrichment and high Il6st expression (Supplementary Fig. 2d).
showed a highly significant decrease in phenotypic LT-HSCs (Fig. 4a), as well as a lower frequency of LSK and LK cells (Fig. 4b, c). The decrease in phenotypic LT-HSCs was accompanied by a loss of LT-HSC quiescence (Fig. 4d, e). In addition, SB treatment reduced the proportion of MkPs and GMPs, whereas preMegEs, preCFU-Es and CFU-Es were all increased (Fig. 4f, g). Finally, the level of circulating platelets was decreased in aged mice upon SB treatment (Fig. 4h). Therefore, inhibition of TGFβR1 signalling counteracted the observed effects of ageing on the HSC, LSK and myelo-erythroid progenitor compartments, and in particular decreased MK/platelet and increased erythroid progenitor output.
TGFβRI inhibition rebalances aged HSC lineage output. Given the link between increased HSC platelet bias and decreased lymphopoiesis during ageing, we investigated whether TGFβ signalling played a role in the age-dependent decrease in lymphoid output. First, we vehicle or SB-treated aged VT/GE mice and performed single cell RNA sequencing of the LSK compartment. Cells were tSNE clustered and their expression of signatures associated with platelet-erythroid (MPP2) and lymphoid-primed MPPs (MPP4) analysed (Fig. 5a, b). We observed a significant decrease in MPP2 priming, and a significant increase in MPP4 priming of LSK cells. The proportion of computationally defined MPP4 cells increased by 21% (from 26% to 32%; \( P = 0.00003; \chi^2\)-test) (Fig. 5c), and showed higher levels of MPP4-specific gene expression (Fig. 5d) after SB treatment. Consistent with this observation, culture of LSK cells from SB-treated mice showed a significant increase in B-cell output when cultured on OP9 stroma (Fig. 5e). This correlated with an increase in the number of clonogenic B-cell progenitors (Fig. 5f), whereas the response of B-cell progenitors to IL-7 was not altered after SB treatment (Supplementary Fig. 4f). To test whether increased lymphopoiesis was due to intrinsic reprogramming of repopulating HSCs we measured the lineage output of transplanted LT-HSCs from vehicle- and SB-treated VT/GE mice. We observed a significant decrease in platelet, erythroid and myeloid output from aged control HSCs, whereas lymphoid output was not affected (Fig. 5g). This led to a general rebalancing of HSC lineage output after SB treatment with highly significant increases in relative B- and T-cell output (Fig. 5h). Overall, whereas aged control HSCs showed clear platelet-lineage bias, output from SB-treated aged HSCs was similar across platelet, erythroid, myeloid and lymphoid lineages, the only significant difference being lower T-cell output (Fig. 5h).

Discussion

We have here used bone marrow stromal cells as unbiased sensors to detect changes to the bone marrow micro-environment during ageing. This approach allowed us to identify molecular signatures associated with IL-6 and TGFβ signalling as upregulated across multiple aged stromal cell types. These results are consistent with previous observations from global molecular profiling of HSCs, which has shown upregulation of inflammation-associated genes in aged HSCs, as well as deregulation of the TGFβ signalling network, and with the finding that levels of IL-6 and TGFβ (this study) are elevated in the bone marrow of aged mice. Therefore, while it is important to note that intrinsic changes to the cell types investigated, including increased expression of Tgfβ1 in aged stromal cells and age-dependent changes to the composition of the HSC compartment, have the potential to influence population-based gene profiling, these observations clearly indicate that signalling through the IL-6 and TGFβ pathways is increased in both hematopoietic and stromal cells in the aged murine bone marrow as a result of increased ligand availability, and identify profiling of stromal cell types as a generally applicable methodology for the identification of micro-environmental changes associated with developmental or pathological processes.

In accordance with previous studies we find that ageing is accompanied by increased levels of MkP and GMP, as well as decreased levels of erythroid and lymphoid progenitor cells. In addition, we show that aged erythroid progenitors are functionally impaired, correlating with a decrease in their expression of erythroid-specific genes. In humans decreased numbers of erythroid colony-forming cells have been observed in anaemic individuals, similarly to what is observed here in aged mice. In a separate study, serum levels of IL-6 were inversely correlated to blood Hb in frail, anaemic individuals. We here observe that antibody-mediated in vivo inhibition of IL-6 leads to recovery of erythroid progenitor function, measured as colony-forming ability of the MPP2, preCFU-E and CD71+ erythroblast populations. In conjunction with our finding that aged HSCs are not impaired in their ability to generate erythrocytes when transplanted into a young BM micro-environment this supports direct inhibition of erythropoiesis by the aged micro-environment as the key component of the age-dependent decline of erythropoiesis, with IL-6 as a putative target for therapeutic intervention. However, while IL-6 inhibition improved the function of aged erythroid progenitors it did not increase their number, indicating that additional micro-environmental factors also play a role. TGFβ1 is likely to contribute, as TGFβRI inhibition increased the number of preMegE, preCFU-E and CFU-E progenitors in aged mice. Inhibition of the TGFβ and Activin signalling improves inefficient erythropoiesis in pathological settings, and our results suggest that this concept may be extended to physiological ageing.

During ageing the hematopoietic system acquires platelet bias, associated with increased MkP and platelet levels. A key driver of this process is a >50-fold expansion of the number of platelet-primed, Vwf+ LT-HSCs, combined with their increased expression of platelet-lineage genes and functional platelet-restriction, as measured by single-cell transplantation. The niches that sustain Vwf+ HSCs are enriched in MKs, and MK-derived TGFβ1 is critical to maintenance of HSC quiescence. We here observe that bone marrow TGFβ1 levels increase with age, correlating with increased HSC platelet bias, and that blocking TGFβRI signalling in aged mice leads to the concomitant loss of HSC platelet bias, decreased HSC quiescence, and declining number and function of HSCs. These observations are consistent with increased local TGFβ1 production by aged non-MK stromal...
cells generating additional niches that maintain quiescent Vwf+ platelet-biased HSCs, and that upon TGFβR1 inhibition these niches are compromised, leading to proliferation and functional depletion of the platelet-biased HSC population. Some cells may retain their HSC immuno-phenotype after their function has been compromised, as previously observed44–46, providing an explanation for the lower overall reconstitution of platelets, erythrocytes and myeloid cells (but not lymphocytes) by TGFβR1 inhibitor-treated HSCs.

In addition to loss of functional HSC platelet bias, TGFβR1 inhibition also improved output of lymphoid progenitors from aged HSCs. In particular, the number of clonogenic B-cell progenitors was increased threefold to 75% of the level in young mice in TGFβR1 inhibitor-treated aged mice, with a similar increase in B- and T-lymphocyte contribution to peripheral blood reconstitution after transplantation of inhibitor-treated HSCs. The functional recovery of lymphopoiesis is accompanied by downregulation of platelet-erythroid (MPP2) and upregulation of lymphoid (MPP4) gene expression in LSK cells. Overall, these results are consistent with gain of lymphoid potential being linked to loss of HSC platelet priming, as previously observed after deletion of FOG-1 from HSCs17.

Our results show that inhibition of IL-6 and TGFβ signalling has the potential to counteract key aspects of hematopoietic...
Fig. 3 IL-6 neutralisation improves aged hematopoiesis. a Bar graph showing the number of genes differentially expressed (>twofold change, adjusted P value <0.05) between young and old stromal cells of the indicated populations. b Heatmap of Metacore Process Networks enriched in three or more aged stromal cell populations at P < 0.005. c Quantification of myelo-erythroid progenitors from old mice treated with control IgG (IgG; N = 9) and neutralising anti-IL-6 antibody (nil-6; N = 10). Data are from five independent experiments. Values are mean ± s.d. *P < 0.05; **P < 0.01; ***P < 0.001 (two-tailed unpaired Student’s t test). Exact P values: MKP: 0.24; preGvM: 0.24; GMP: 0.03; preMegE: 0.02; preCFU-E: 0.82; CFU-E: 0.85. d In vitro colony-forming potential of LK (defined as Lin−/Sca-1−/c-Kit+) cells isolated from BM of old mice after injection of IgG isotype control (N = 4) or nil-6 Ab (N = 4) assayed under E condition (M3436; 500 cells). Data are from two different experiments. Values show mean ± s.e.m. ***P < 0.001 (two-tailed unpaired Student’s t test). Exact P value: 0.0005. e In vitro colony-forming potential of preCFU-E cells isolated from BM of old mice after injection of IgG isotype control (N = 4) or nil-6 Ab (N = 4) assayed under GM condition (M3354; 500 cells). Data are from two different experiments. Values show mean ± s.e.m. **P < 0.01 (two-tailed unpaired Student’s t test). Exact P value: 0.0005. f In vitro colony-forming potential of preCFU-E cells isolated from BM of old mice after injection of nil-6 Ab (N = 4) assayed under E condition (M3436; 500 cells). Data are from two different experiments. Values show mean ± s.e.m. ***P < 0.001 (two-tailed unpaired Student’s t test). Exact P value = 0.0005. g GSEA analysis comparing expression of preCFU-E-specific genes in preCFU-E isolated from young and old IL-6-treated mouse cells. The normalised enrichment score (NES) and P value are shown. h GSEA analysis comparing expression of preCFU-E-specific genes in preCFU-E isolated from young and old anti-IL-6-treated CFU-Es. The normalised enrichment score (NES) and P value are shown. Source data are provided as a source data file.

ageing. In particular, we find that TGFβ signalling is critical to the maintenance of age-associated HSC platelet-myeloid lineage bias, and that its inhibition improves lymphoid output from HSCs. In young mice myeloid and erythroid-lineage output from fate-restricted HSCs are correlated. However, during ageing erythropoiesis decreases while myeloid output is increased. We here find that increased TGFβ and IL-6 signalling impair the generation and function of erythroid-lineage progenitors, respectively, providing a mechanism for the distinct effects of ageing on erythroid and myeloid lineage output. Together, these findings identify druggable targets with the potential to improve aged hematopoiesis and in particular show that targeting multiple signalling systems may be required to normalise aged erythropoiesis.

Methods

Animals. All experimental procedures and mouse breeding and maintenance were in accordance with UK Home Office regulations. All experiments were approved by the Oxford University Clinical Medicine Ethical Review Committee. All mice used were from the C57Bl/6 background. For transplantsations recipient and competitor cells were of the CD45.1 allotype. Donor mice of the CD45.2 allotype and carried the VwfdTomato and Gata1-EFP transgenes. Young mice were 2–3 months old and old mice were 24–25 months old. Only female mice were used for experiments. When multiple experimental groups were analysed, mice were allocated so that each group was evenly matched for age range. No statistical methods were used to predetermine the experimental sample size.

HSC transplantation. Fifty phenotypically defined LT-HSCs (LSKCD48-CD130+CD34−) were isolated from young (2–3 months) or old (24–25 months) VwfΔTomato/Gata1-EFP (CD45.2) double reporter mice and intravenously injected along with 2 x 105 young wild-type unfractionated C57Bl/6 bone marrow cells (CD45.1) into lethally irradiated CD45.1 young recipients (2 x 500 rad). Peripheral blood reconstitution analysis was performed at 16 weeks after transplantation.

In vivo inhibitor studies. Aged mice were injected three times intraperitoneally (i.p.) with 100 µg of mouse IL-6 neutralising antibody or Rat IgG2a isotype control (InvivoGen) dissolved in 1 mL of sterile saline solution at 1-week intervals and the mice were killed for analysis after 6 days from the last injection. For SB-525334 treatment, aged mice were treated for 13 consecutive days, once daily, with either 30 mg/kg SB-525334 dissolved in corn oil or vehicle (corn oil alone) via oral gavage. The mice were culled for analysis the day following the last gavage.

Hydrodynamic injection. Krebs–Ringer modified buffer (pH 7.4) with 0.05% BSA was used as final working buffer for all hydrodynamic injections as previously described. The injection volume was -8–12% of the mouse body weight. After being generally anaesthetised, mice were tail vein-injected in the working buffer with 1 µg of a pCMV6-Entry vector containing IL-6 CDNA (OriGene, cat no. MR227281) or an empty pCMV6-entry vector (OriGene, cat no. PS000001). The injected mice were incubated for 7 days before being analysed. Blood samples were collected into eppendorf tubes and left at room temperature to clot for 2 h. The samples were then centrifuged (20 min, 2000 × g, 4 °C) and the supernatant was collected and stored at −80 °C immediately. IL-6 serum levels were determined using the IL-6 ELISA Kit (BioLegend, cat no. 431301).

TGfβ1 ELISA. Femurs and tibias from mice were flushed with 200 µL of PBS; the flushed BM suspension was centrifuged (5 min, 500 × g, 4 °C) and the BM supernatant was collected. TGfβ1 bone marrow supernatant levels were determined using the Quantikine ELISA Kit (R&D systems, MBBF00) according to the manufacturer’s instructions.

Peripheral blood parameters measurement. Peripheral blood from young and old mice was collected into lithium heparin-coated microvrettes (Sarstedt) and diluted five times in PBS. White blood cells (WBC), Red blood cells (RBC) and platelet counts were measured using a Sysmex KX-21N Automated Haematology Analyser (three replicates/sample). Hb and HCT values were obtained in the same way (three replicates/sample).

Flow cytometry and cell sorting. Details of mouse antibodies and viability dyes used for each staining panel are shown in Supplementary Data 3. All antibodies were used at optimal working concentrations, determined by titration. Hematopoietic stem and progenitor cells, myelo-erythroid progenitor cells, peripheral blood WBC, RBC and PLT were analysed as previously described. The optimal gating strategy was chosen using a combination of fluorescence minus one controls and populations known to be negative/positive for the antigen. Cell acquisition and analysis were performed using LSRi or LSR Fortessa X-20 cytometer (BD Biosciences) using BD FACSDivA software (BD Biosciences). Phenotypically defined LT-HSCs, LK, LSK and preCFU-E were sorted using a BD FACSaria II cell sorter (BD Biosciences). Analysis was performed using Flowjo software version 10.3 (Flowjo LLC, OR, USA).

HSC cell cycle analysis. For HSC cell cycle analysis, BM cells were fixed and permeabilized using BD cytofix/cytperm fixation and permeabilization solution (BD Biosciences; BD 554714) following initial Fe-cell-block incubation. Fixed cells were then stained with Ki-67 PE (BD Biosciences; BD 556027) overnight. The day after, cells were washed and stained with 4′,6-diamidino-2-phenylindole (0.5 mg/mL) (ThermoFisher Scientific, Waltham, MA, USA) for 1 h before acquisition. Cell acquisition and analysis were performed using LSRii or LSR Fortessa X-20 cytometer (BD Biosciences) using BD FACSDivA software (BD Biosciences). Analysis was performed using Flowjo software version 10.3 (Flowjo LLC, OR, USA).

Early B precursor isolation. BM cells were stained with biotinated antibody B220 Biotin (eBioscience, 13–0452–82) following initial Fe-cell-block incubation. After incubation, cells were washed twice with PBS/5% FCS, centrifuged and incubated with Anti-Biotin Microbeads (Miltenyi Biotec, 130–090–485). Magnetic purification was performed according to the manufacturer’s instructions. B220+ cells were counted and stained with SAV-Brilliant Violet 605 (BioLegend, 450329) and anti-Mouse IgM PE-Cyanine7 Antibody (Life Technologies, 25–5790–81). After wash, cells were stained with 7-Aminomethylcoumarin D (7AAD; Biotium 40037) and phenotypically defined early B precursors (7AAD−B220−IL-7−) sorted using a BD FACSaria II cell sorter (BD Biosciences). Analysis was performed using Flowjo software version 10.3 (Flowjo LLC, OR, USA).

B-cell progenitor proliferation assay. Proliferation was measured using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS) (Promega, G3582) following the manufacturer’s instructions. In detail, phenotypically defined early B precursors (100,000 cells/well) were sorted in 96-well flat-bottom plates in 0.1 mL Opti-Mem supplemented with 10% FBS, penicillin-streptomycin (100 U/mL) and 2 mM (2×) G2-mercaptoethanol (50 µM) using a BD FACSaria II cell sorter (BD Biosciences). mL−7 (R&D Systems) was added at different concentrations (0.1–1–10–100 ng/mL). After 48 h, 20 µL of CellTiter 96® Aqueous One
Solution Reagent was added into each well of the 96-well assay plate containing the samples, plates were incubated at 37 °C for 4 h in a humidified, 5% CO₂ atmosphere and the absorbance at 490 nm was recorded.

**In vitro erythroid/myeloid colony-forming assay.** Media used for erythroid/myeloid colony-forming assays were from STEMCELL Technologies and were used according to the manufacturer’s instructions. Overall, 500 cells were seeded per culture in M3436 (supplemented with SCF 100 ng/ml) and M3534, and the colonies generated in methylcellulose were scored by morphology as CFU-E and CFU-GM, respectively after 7 days. Cells were plated in triplicate for each assay.

**In vitro lymphoid colony-forming assay.** Media used for lymphoid colony-forming assay was from STEMCELL Technologies and was used according to the manufacturer’s instructions. In total, 25,000 phenotypically defined early B precursors (7AAD⁻ B220⁺ IgM⁻) were seeded per culture in M3630 (supplemented with SCF 20 ng/ml) and the colonies generated in methylcellulose were scored by morphology after 7 days. Cells were plated in triplicate.
Fig. 4 Effect of TGFβRI inhibition on aged hematopoiesis. Bar graphs showing the number of LT-HSCs (a), LSK (b) and LC (c) cells in bone marrow of old mice treated with vehicle (VEH) or SB (LT-HSCs and LSK: FCS buffer, aspirated and passed through a 50-μm filter into a 50-mL Falcon tube and stored at 4 °C. Collagenase treatment was repeated once more on the remaining bone material to ensure maximum niche cell recovery. The resulting cell solution was washed with 10 mL PBS/5% FCS and counted. Cells were then stained for FACS using the antibodies outlined in Supplementary Data 3.

Generation of cdNA libraries using Smart-seq2 protocol. An adapted version of the low-cell number SMART-seq protocol35,54 was used for processing bulk RNA-seq samples. For all bulk RNA-seq experiments, 100 cells/biological replicate were isolated by FACS directly into thin-walled PCR tubes containing 4 μl of lysis buffer containing: 0.2% Triton X-100 (Sigma Aldrich), for 45 min at 37 °C on a rotating plate. Digested bone fragments were allowed to settle and the top layer was aspirated, passed through a 70-μm mesh filter into a 50 mL Falcon tube and stored at 4 °C. Collagenase treatment was performed for 4 weeks before being further processed. For the reverse transcriptase, 6 μl of the following mix was added: 2 μl SuperScript II first strand buffer, 0.5 μl DTT (100 mM), 2 μl Betaine (5 M), 0.1 μl MgCl2 (1 M), 0.25 μl RNAse Inhibitor (40 U/μl), 0.1 μl TSO (100 μM), 0.25 μl SuperScript II RT (200 U/μl) and 0.4 μl water. After reverse transcriptase, 15 μl was added containing 12.5 μl Kapa HiFi HS ReadyMix (2x) and 0.125 μl ISPCR primers (10 μM). The five thermal conditions for RT and pre-amplification were according to the original Smart-seq2 protocol35,54. The number of cycles used for PCR amplification was 18. After PCR amplification, cdNA libraries were purified using Ampure XP magnetic beads according to the manufacturer’s instructions. After purification, the libraries were resuspended in 17.5 μl of buffer EB (Qiagen) and stored at −20 °C. Quality and concentration of the cdNA libraries generated was assessed using High-Sensitivity Bioanalyzer (Agilent Technologies).

Illumina library preparation and sequencing. In total, 0.7 ng of cdNA was amplified using the Nextera XT DNA Sample Preparation Kit (Illumina) according to the manufacturer’s instructions, except that one-fourth of the volumes indicated were used. Purification of the product was done with a 1:1 ratio of AMPure XP beads, with a final elution in 17.5 μl of resuspension buffer provided from the Nextera kit. Samples were loaded on a High-Sensitivity DNA chip (Agilent Technologies) to check the size and quality of the indexed library, and the concentration was measured with the Qubit High-Sensitivity DNA kit (Invitrogen). Libraries were pooled to a final concentration of 4 nM and were sequenced on Illumina NextSeq 500 (76 bp single-end) or Illumina HiSeq4000 (50 bp single-end) after preparation according to manufacturer’s instructions.

Data analysis. Bulk RNA-seq samples were sequenced on Illumina NextSeq 500 (76 bp, single-end) or Illumina HiSeq4000 (50 bp, single-end) platforms. FASTQ files were inspected using FastQC v0.11.1 (followed by Nextera adaptors sequence removal using TrimGalore! (v0.4.1; http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) with a stringency setting of 3. Reads were aligned to the mm9 transcriptome using STAR (v2.4.2a)55. Unique reads were counted using featureCounts (v1.4.5-p1)56 and the UCSC mm9 annotation project. Reads were imported into R Studio and a local method was used for differential gene expression analysis. Counts were normalised using the rlog Transformation method was used for differential gene expression analysis. Counts were normalised using the rlog Transformation method was used for differential gene expression analysis. Counts were normalised using the rlog Transformation method was used for differential gene expression analysis. 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Ranked DEGs (adjusted \( P \leq 0.05 \)) were used as input for Metacore, which models functionally enriched ontology based on a hypergeometric distribution model. After calculating a \( P \) value for all terms within a given ontology, each term is then tested as a separate hypothesis which results in the generation of a \( q \) value. This subsequently provides an estimate of false discovery rate (FDR). All Metacore output displayed in this study have both \( P \leq 0.05 \) and FDR \( \leq 0.05 \). GSEA (v3.060) was performed using the javaGSEA application. RPKM values were imported as input data. Gene sets for GSEA were taken from MSigDB (IL-6 gene set M14344) and Mancini et al.61.

Single cell sequencing library preparation. LSK cells were FACS isolated from aged (24–25 months old) mice treated with vehicle or SB-525334 and processed using the 10\( \times \) Genomics droplet-based sampler. Library preparation was performed following the Chromium Single Cell 3’ Reagents Kit v2 users guide (CG00052, Rev C). Quantification of cDNA was done using the Qubit dsDNA HS kit (Invitrogen) and a Qubit fluorometer (Invitrogen). Fragment sizes were determined using an Agilent high-sensitivity DNA chip (Agilent Technologies) and an Agilent 2100 Bioanalyzer (Agilent Technologies). After pooling, library quantification was determined using the KAPA qPCR platform (Kapa Biosystems).

10\( \times \) Chromium data analysis. Alignment, quality checking, filtering and analysis of UMI-based scRNA-seq datasets was achieved using the following steps. Demultiplexing and alignment: Sequencing of UMI-based cDNA libraries from young and aged unfractionated LSK was carried out using the Illumina HiSeq6000 platform (75 bp paired-end). Sample demultiplexing was completed by the Wellcome Trust Centre for Human Genetics, University of Oxford, and FASTQ files.
were aligned to the mm10 reference genome using CellRanger (v2.0.1; 10× Genomics). Biological replicates from the same conditions were aggregated using the CellRanger::Aggregate function. Filtering, merging and normalisation data: Filtered gene-cell matrices of aggregated samples were imported into R Studio using the Seurat::Read10x function (v2.3.0). SNPE was run to visualise the gene expression of single cells in two-dimensional space. Differential gene expression analysis was carried out using a negative binomial statistical test56. DEGs with an adjusted P < 0.05 and LogFC > 0.25 were returned and used for subsequent analyses. GSEA60 was used to determine enriched functions. DEGs were ranked by function and the MPP4 gene set defined above, with MPP4 cells defined as those scoring above the cutoff that identified the same proportion of MPP4 cells in young mice as flow cytometry59.

Statistical analysis. Statistical comparisons of continuous data with assumed normal distribution (mean percentages) were performed with parametric unpaired two-tailed Student’s t-test. Two-sided Mann–Whitney U-test was used for data with non-normal distribution. Statistical comparisons of categorical data (frequencies) were performed with nonparametric two-tailed P-test. Student’s t-test, Mann–Whitney U-test and P-test were performed with GraphPad Prism software or in R using the appropriate R function. Only statistically significant differences (P < 0.05) are indicated in the figures.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The RNA sequencing data generated have been deposited in the GEO database under the accession code GSE130899. Further details are available from the corresponding authors upon reasonable request. Source Data are provided with this paper.

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