Inhibitory receptors bind ANGPTLs and support blood stem cells and leukaemia development

Junke Zheng1,2,*, Masato Umikawa1,3,*, Changhao Cui1,*, Jiuyuan Li1, Xiaoli Chen1, Chaozheng Zhang1, HoangDinh Huynh1, Xunlei Kang1, Robert Silvany1, Xuan Wan1, Jingxiao Ye1, Alberto Puig Cantó1, Shu-Hsia Chen5, Huan-You Wang6, E. Sally Ward4 & Cheng Cheng Zhang1

How environmental cues regulate adult stem cell and cancer cell activity through surface receptors is poorly understood. Angiopoietin-like proteins (ANGPTLs), a family of seven secreted glycoproteins, are known to support the activity of haematopoietic stem cells (HSCs) in vitro and in vivo1–10. ANGPTLs also have important roles in lipid metabolism, angiogenesis and inflammation, but were considered ‘orphan ligands’ because no receptors were identified11–12. Here we show that the immune-inhibitory receptor human leukocyte immunoglobulin-like receptor B2 (LILRB2) and its mouse orthologue paired immunoglobulin-like receptor (PIRB) are receptors for several ANGPTLs. LILRB2 and PIRB are expressed on human and mouse HSCs, respectively, and the binding of ANGPTLs to these receptors supported ex vivo expansion of HSCs. In mouse transplantation acute myeloid leukaemia models, a deficiency in intracellular signalling of PIRB resulted in increased differentiation of leukaemia cells, revealing that PIRB supports leukaemia development. Our study indicates an unexpected functional significance of classical immune-inhibitory receptors in maintenance of stemness of normal adult stem cells and in support of cancer development.

We used multiple approaches, including expression cloning, to identify the receptor(s) for ANGPTLs. Human LILRB2, when ectopically expressed on Ba5 cells, enabled the cells to specifically bind glutathione S-transferase (GST)–ANGPTL5 as determined by flow cytometry (Fig. 1a). LILRB2 is a member of the immune-inhibitory B-type subfamily of LILR receptors13 and contains four immunoglobulin domains and three immunoreceptor tyrosine-based inhibitory motifs. Using flow cytometry analysis, we further demonstrated that LILRB2-overexpressing 293T cells demonstrated enhanced binding to several ANGPTLs, especially ANGPTL2 and GST–ANGPTL5 (Fig. 1b and Supplementary Fig. 1a, b). ANGPTL2 and GST–ANGPTL5 also bound to LILRB3- and LILRB5-overexpressing cells, although with a lower affinity than to LILRB2-expressing cells (Supplementary Table 1). In addition, ANGPTL1 and ANGPTL7 bound to 293T cells overexpressing LAIR1 (ref. 14). (Supplementary Table 1 and Supplementary Fig. 2). ANGPTLs did not bind to LILRAs, LILRB1 or LILRB4 (Supplementary Table 1).

Because ANGPTL2 and GST–ANGPTL5 bound to LILRB2-expressing cells better than other ANGPTLs, we further assessed the molecular interaction between ANGPTL2/ANGPTL5 and LILRB2.

![Cell-surface LILRB2 binds to ANGPTLs.](image)

**Figure 1 | Cell-surface LILRB2 binds to ANGPTLs.** a, Flow cytometry analysis of GST–ANGPTL5–Flag binding to uninfected Ba5 cells or MSCV–GFP, MSCV–Tie-2–GFP or MSCV–LILRB2–GFP stably infected Ba5 cells. Mean fluorescence intensities are indicated. b, Flow cytometry analysis of indicated Flag-tagged ANGPTLs binding to LILRB2-transfected 293T cells. c, ANGPTL2 and ANGPTL5 bound to the ECD of LILRB2 but not Tie-2 in conditioned medium (CM) of cotransfected 293T cells. d, e, Concentration-dependent specific (d) and competitive (e) GST–ANGPTL5 binding to LILRB2 stably expressed Ba5 cells (n = 3). Error bars denote s.e.m. IB, immunoblotting.

---

1Departments of Physiology and Developmental Biology, University of Texas Southwestern Medical Center, Dallas, Texas 75390, USA. 2Key Laboratory of Cell Differentiation and Apoptosis of Chinese Ministry of Education, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China. 3Department of Medical Biochemistry, University of the Ryukyus, Okinawa 903-0215, Japan. 4Department of Immunology, University of Texas Southwestern Medical Center, Dallas, Texas 75390, USA. 5Department of Oncological Sciences, Mount Sinai School of Medicine, New York, New York 10029-6574, USA. 6Department of Pathology, University of California San Diego, La Jolla, California 92093, USA.

*These authors contributed equally to this work.
Co-transfection of ANGPTL2 or ANGPTL5 with LILRB2 extracellular domain (ECD) fused to human IgG–Fc (LILRB2–Fc) into 293T cells followed by immunoprecipitation and western blot showed that both ANGPTL2 and ANGPTL5 interacted with the ECD of LILRB2, but not that of Tie-2 (Fig. 1c and Supplementary Fig. 1c). The direct interactions between ANGPTLs and LILRB2 were confirmed by in vitro coimmunoprecipitation, using purified ANGPTL2–Flag or GST–ANGPTL5 and LILRB2–Fc (Supplementary Fig. 1d) and by surface plasmon resonance (SPR; Supplementary Fig. 3). A liquid-phase binding assay with 125I-labelled GST–ANGPTL5 demonstrated that the interaction between ANGPTL5 and cell-surface LILRB2 was specific and saturable, with half maximal saturation of the interaction as 5.5 ± 1.1 nM (Fig. 1d, e). Although untagged ANGPTLs bind to LILRB2, the type or the position of tagging could affect the binding (Supplementary Table 2).

Because several ANGPTLs support expansion of HSCs4–10, we sought to determine whether ANGPTLs bound to LILRB2 or LAIR1 on primary human cord blood cells. Flow cytometry analysis showed that ANGPTL 1, 2, 5 and 7 all bind to LILRB2 on human cord blood cells, and that ANGPTL2 and GST–ANGPTL5 had higher affinities (Fig. 2a, Supplementary Fig. 4 and Supplementary Table 1). The binding of ANGPTL1 and ANGPTL7 to LAIR1+ human cord blood cells was relatively weak (Supplementary Fig. 5), and we therefore focused on studying the binding of ANGPTL2 and ANGPTL5 to LILRB2 in subsequent experiments.

We determined whether LILRB2 was expressed on human HSCs. Flow cytometry and quantitative (q)RT–PCR analyses showed that LILRB2 was expressed on the surface of 40–95% of human cord blood CD34+ CD38− CD90+ cells (95% in the experiment shown in Fig. 2b and Supplementary Fig. 6); this population is enriched for HSCs.

GST–ANGPTL5 treatment induced increased phosphorylation of calcium/calmodulin-dependent protein kinase (CAMK)-2 and -4 in human cord blood mononuclear cells (Supplementary Fig. 7). It is of note that CAMK4 is required for maintenance of the potency of HSCs10. Suppression of LILRB2 expression with short hairpin RNAs effectively reduced ANGPTL binding (Supplementary Fig. 8). Importantly, the silencing of LILRB2 resulted in decreased repopulation of human cord blood HSCs as measured by reconstitution analysis in non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice (1% repopulation from cultured knockdown cells compared to 15% repopulation from cultured normal cells in medium STFA5; Fig. 2c). Together, these data indicate that ANGPTL5 supports expansion of human cord blood HSCs in a process at least partially mediated by the surface receptor LILRB2.

PIRB is the mouse membrane orthologue of human LILRBs16,17. ANGPTL2, ANGPTL3 and GST–ANGPTL5 bound to PIRB as determined by flow cytometry (Fig. 3a and Supplementary Fig. 9) and coimmunoprecipitation (Fig. 3b and Supplementary Fig. 10). As with human cord blood HSCs, mouse HSCs were also enriched for PIRB expression (Fig. 3c and Supplementary Fig. 11).

To study the function of PIRB in mouse HSCs, we used PIRB-deficient (PIRBTM) mice18, in which four exons encoding the transmembrane domain and part of the intracellular domain were deleted. PIRBTM cells freshly isolated from 3-week-old mice had significantly decreased CAMK4 phosphorylation, and binding of ANGPTL to PIRB induced phosphorylation of PIRB, recruitment of SHP-1 and SHP-2 (also known as PTPN6 and PTPN11, respectively) and CAMK4 activation (Supplementary Figs 12 and 13). These results suggest that certain ANGPTLs may be the ligands of PIRB that activate CAMK4 in vivo.

![Figure 2](image-url)  

**Figure 2** | LILRB2 mediates the effect of ANGPTL in supporting the repopulation of human cord blood HSCs.  

**a.** Flow cytometry analysis of indicated Flag-tagged ANGPTLs binding to LILRB2+ human cord blood mononuclear cells (FACSAria). Mean fluorescence intensities are indicated.  

**b.** Representative flow cytometry plots for the co-staining of CD34, CD38, CD90 and LILRB2 in human cord blood mononuclear cells (FACSCalibur).  

**c.** Human cord blood CD34+ cells infected with LILRB2 shRNA-encoding virus (KD) or control (Ctrl) scramble shRNA virus were transplanted into sublethally irradiated NOD/SCID mice before or after culture for 10 days. SCF+TPO+Flt3L (STF) or STF+ANGPTL5 (STFA5) was used in the culture. Shown is the human donor repopulation after 2 months (n = 5–11). *P < 0.05. Error bars denote s.e.m.
Because SHP-2 and CAMK4 are required for the repopulation of HSCs, and the chemical inhibition of CAMK2, a homologue of CAMK4, induces differentiation and suppresses proliferation of myeloid leukaemia cells, we sought to determine whether PIRB was important for HSC activity. Although the adult PIRBTM mice have certain immune and neuronal defects, they are grossly normal in haematopoiesis. Interestingly, competitive repopulation showed that PIRBTM fetal liver HSCs had approximately 50% decreased repopulation activity (Supplementary Fig. 14). Moreover, although ANGPTL2 and ANGPTL5 had little effect on ex vivo expansion of adult PIRBTM HSCs, they supported ex vivo expansion of adult wild-type HSCs (Fig. 3d and Supplementary Fig. 14), as we previously demonstrated. Collectively, our results indicate that ANGPTLs bind human LILRB2 and mouse PIRB to support HSC repopulation.

On the basis of our in silico analysis of a pool of 9,004 samples described previously, the level of LILRB2 messenger RNA is at least fourfold higher in the human acute monoblastic and monocytic leukaemia cells (M5 subtype of acute myeloid leukaemia (AML)) than in other AML cells (Supplementary Fig. 15). As human acute monoblastic and monocytic leukaemia cells are often associated with rearrangement of mixed-lineage leukaemia (MLL; a histone methyltransferase deemed a positive global regulator of gene transcription), we used a retroviral MLL–AF9 transplantation mouse model to further examine the role of PIRB in regulation of AML development. Wild-type or PIRBTM donor Lin<sup>−</sup> Mac-1<sup>−</sup> Kit<sup>−</sup> cells in both MLL–AF9-transduced wild-type and PIRBTM donor cells formed mostly compact colonies, whereas PIRBTM cells tended to form more diffuse colonies (Fig. 4e). The formation of diffuse colonies indicates high differentiation potential. The inhibition of differentiation of AML cells by PIRB is in accordance with previous reports that PIRB inhibits differentiation of myeloid-derived suppressive cells and osteoclasts, as well as our data showing that endogenous ANGPTLs inhibit differentiation and increase replating efficiency of haematopoietic progenitors (Supplementary Fig. 17). Moreover, PIRBTM primary colony-forming units were unable to form secondary colonies upon replating (Fig. 4f), indicating that PIRB supports self-renewal of AML c.f.u. cells.

Finally, we analysed the molecular signalling triggered by the binding of ANGPTLs to PIRB in AML cells. PIRBTM AML cells had decreased phosphorylation of phosphatase SHP-2 (Supplementary Fig. 13d), which is known to be associated with LILRB receptors and is an oncogene that supports leukaemia development. ANGPTLs also stimulate SHP-2 phosphorylation (Supplementary Fig. 13d). Similar to untransformed PIRBTM cells, PIRBTM AML cells had decreased CAMK4 activation (data not shown). Furthermore, wild-type Mac-1<sup>−</sup> Kit<sup>−</sup> cells had much greater expression of leukaemia initiation/maintenance genes, but markedly decreased expression of myeloid-differentiation genes as determined by DNA microarray analyses (Fig. 4g). qRT–PCR confirmed the increased expression of several HOXA genes, Mye1, Eya1, Myb and Mef2c in wild-type Mac-1<sup>−</sup> Kit<sup>−</sup> cells than their PIRBTM counterparts (Supplementary Fig. 18); these genes are critical for initiation or maintenance of MLL-rearranged cells. Isotype control is indicated in the left panel. D. Competitive reconstitution of 8-day cultured progenies of input equivalent to 250 Lin<sup>−</sup> Sca-1<sup>−</sup> Kit<sup>−</sup> CD34<sup>+</sup> Flk-2<sup>−</sup> bone marrow HSCs from wild-type (WT) or PIRBTM donors (n = 5). SCF, TPO and FGF-1, with or without ANGPTL2, were used in culture. cHSCs, cultured HSCs. P = 0.05. Error bars denote s.e.m.
and exhaustion. Adult stem cells and cancer cells probably require inflammatory ANGPTLs and protecting HSCs from excessive activation function as sensors of inflammation through binding to the inflammatory context. 

Comparison of the sizes of spleen, liver and numbers of peripheral blood (PB) cells of the mice transplanted with wild-type (WT) MLL–AF9 cells and PIRBTM MLL–AF9 cells at 28 days after transplantation (n = 6). d, Representative flow cytometry plots showing that PIRBTM AML mice have decreased Mac-1 cells of the mice transplanted with wild-type (WT) MLL–AF9 cells and PIRBTM infected WT or PIRBTM haematopoietic progenitors (8,17). It would be important to investigate the in vivo context in which these different ligands bind LILRB and induce signalling. As ANGPTLs can be abundantly expressed by many types of cells, including those from endocrine organs and potential bone marrow niche (endothelium and adipocytes), and can be induced by hypoxia, these secreted factors may have important direct and indirect effects on the activities of HSCs and leukemia stem cells in vivo. Although the LILRB/PIRB receptors were reported to suppress activation of differentiated immune cells and inhibit neutrole outgrowth of neural cells (8,17), they support HSC repopulation and inhibit differentiation of AML cells. This result suggests the importance of these ‘inhibitory receptors’ in maintenance of stemness of normal stem cells and support of leukemia development. In contrast to the ‘stimulatory receptors’ such as interferon receptors or Toll-like receptors that activate and induce differentiation of HSCs upon inflammation, LILRB2 and PIRB may function as sensors of inflammation through binding to the inflammatory ANGPTLs and protecting HSCs from excessive activation and exhaustion. Adult stem cells and cancer cells probably require both stimulatory receptors and inhibitory receptors to maintain the balance of their cell fates.

METHODS SUMMARY

Plasmid cytomegalovirus (CMV)–Kozak human Angiopoietin-1 and ANGPTL 1, 2, 3, 4, 6 and 7 with Flag tags at the carboxy terminus were used for transfection. ANGPTL2–Flag was purified using M2 resin. Purified GST–ANGPTL5 was purchased from Abnova. Bacterially expressed Flag-ANGPTL2 (with Flag at the amino terminus) and ANGPTL2–Flag (with Flag at the C terminus) were constructed in pET-28b (+) vector, and GST–ANGPTL2–Flag in pGEX vector, and expressed and purified from bacteria. Murine stem cell virus (MSCV)–E1–E3–green fluorescent protein (GFP) or control retrovirus-infected Ba5 cells, CMV–driven LILRA–, LILRB–, PIRB–, or LAIR1–transfected 293T cells, or human mononuclear cord blood cells were used in binding assays. See the Methods for detailed experimental methods for flow cytometry, coimmunoprecipitation, SPR, liquid-phase binding, culture, transplantation, c.f.u. and gene set enrichment analysis (GSEA). Mice were maintained at the University of Texas Southwestern Medical Center animal facility. All animal experiments were performed with the approval of University of Texas Southwestern Committee on Animal Care.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.
2. Zhang, C. C. et al. Angiopoietin-like proteins stimulate ex vivo expansion of hematopoietic stem cells. Nature Med. 12, 240–245 (2006).
3. Zhang, C. C. & Lodish, H. F. Cytokines regulating hematopoietic stem cell function. Curr. Opin. Hematol. 15, 307–311 (2008).
4. Huynh, H. et al. IGFBP2 secreted by a tumorigenic cell line supports ex vivo expansion of mouse hematopoietic stem cells. Stem Cells 26, 1628–1635 (2008).
5. Zhang, C. C. & Lodish, H. F. Cytokines regulating hematopoietic stem cells. Proc. Natl Acad. Sci. USA 107, 7799–7804 (2010).
6. Lin, M. & Zon, L. L. Genetic analyses in zebrafish reveal that angiopoietin-like proteins 1 and 2 are required for HSC development during embryogenesis. Am. Soc. Hematol. 50th Ann. Meeting Abstract 729 186 (2008).
7. Khoury, M. et al. Mesenchymal stem cells secreting angiopoietin-like-5 support efficient expansion of human hematopoietic stem cells without compromising their repopulating potential. Stem Cells Dev. 20, 1371–1381 (2011).
8. Drake, A. C. et al. Human CD34+CD133+ hematopoietic stem cells cultured with growth factors including Angptl5 efficiently engraft adult NOD-SCID II zyg (NSG) mice. PloS ONE 6, e18382 (2011).
9. Zheng, J., Huynh, H., Umita, M., Silvany, R. & Zhang, C. C. Angiopoietin-like protein 3 supports the activity of hematopoietic stem cells in the bone marrow niche. Blood 117, 470–479 (2011).
10. Zheng, J. et al. Ex vivo expanded hematopoietic stem cells overcome the MHC barrier in allologeneic transplantation. Cell Stem Cell 9, 119–130 (2011).
11. Hato, T., Tabata, M. & Oike, Y. The role of angiopoietin-like proteins in angiogenesis and metabolism. Trends Cardiovasc. Med. 18, 6–14 (2008).
12. Tabata, M. et al. Angiopoietin-like protein 2 promotes chronic adipose tissue inflammation and obesity-related systemic insulin resistance. Cell Metab. 10, 178–188 (2009).
13. Barrow, A. D. & Trowsdale, J. The extended human leukocyte receptor complex: diverse ways of modulating immune responses. Immunol. Rev. 224, 98–123 (2008).
14. Mayeaud, L. LAIR and collagens in immune regulation. J. Biol. Chem. 280, 33101–33108 (2005).
15. Takai, T., Nakamura, A. & Endo, S. Role of PIR-B in autoimmune glomerulonephritis. J. Biomed. Biotechnol. 2011, 275302 (2011).
16. Atwal, J. K. et al. PIR-B is a functional receptor for myelin inhibitors of axonal regeneration. Science 322, 967–970 (2008).
17. Syken, J., Grandpre, T., Kanold, P. O. & Shatz, C. J. PIR-B restricts ocular-dominance plasticity in visual cortex. Science 313, 1795–1800 (2006).
18. Chen, R. J. et al.SHIP-2 heterozygous hematopoietic stem cells have deficient repopulating ability due to diminished self-renewal. Exp. Hematol. 34, 1229–1238 (2006).
19. Si, J. & Collins, S. J. Activated Ca2+ /calmodulin-dependent protein kinase II is a critical regulator of myeloid leukemia cell proliferation. Cancer Res. 68, 3733–3742 (2008).
20. Lukk, M. et al. A global map of human gene expression. Nature Biotechnol. 28, 322–324 (2010).
21. Atwal, J. K. et al. Transformation from committed progenitor to leukemia stem cell initiated by MLL–AF9. Nature 442, 818–822 (2006).
22. Shatz, C. J. et al. Identification and characterization of leukemia stem cells in murine MLL–AF9 acute myeloid leukemia. Cancer Cell 10, 257–268 (2006).
23. Lavau, C., Zilvassy, S. J., Stanl, R. & Cleary, M. L. Immortalization and leukemic transformation of a myelomonocytic precursor by retrovirally transduced HRX-ENL. EMBO J. 16, 4226–4237 (1997).
24. Ma, G. et al. Paired immunoglobulin-like receptor-B regulates the suppressive function and fate of myeloid-derived suppressor cells. Immunity 34, 385–395 (2011).
25. Meyaard, L. LAIR and collagens in immune regulation. J. Biol. Chem. 280, 33101–33108 (2005).
26. Lin, M. & Zon, L. L. Genetic analyses in zebrafish reveal that angiopoietin-like proteins 1 and 2 are required for HSC development during embryogenesis. Am. Soc. Hematol. 50th Ann. Meeting Abstract 729 186 (2008).
27. Khoury, M. et al. Mesenchymal stem cells secreting angiopoietin-like-5 support efficient expansion of human hematopoietic stem cells without compromising their repopulating potential. Stem Cells Dev. 20, 1371–1381 (2011).
28. Drake, A. C. et al. Human CD34+CD133+ hematopoietic stem cells cultured with growth factors including Angptl5 efficiently engraft adult NOD-SCID II zyg (NSG) mice. PloS ONE 6, e18382 (2011).
29. Zheng, J., Huynh, H., Umita, M., Silvany, R. & Zhang, C. C. Angiopoietin-like protein 3 supports the activity of hematopoietic stem cells in the bone marrow niche. Blood 117, 470–479 (2011).
30. Zheng, J. et al. Ex vivo expanded hematopoietic stem cells overcome the MHC barrier in allologeneic transplantation. Cell Stem Cell 9, 119–130 (2011).
31. Hato, T., Tabata, M. & Oike, Y. The role of angiopoietin-like proteins in angiogenesis and metabolism. Trends Cardiovasc. Med. 18, 6–14 (2008).
32. Tabata, M. et al. Angiopoietin-like protein 2 promotes chronic adipose tissue inflammation and obesity-related systemic insulin resistance. Cell Metab. 10, 178–188 (2009).
33. Barrow, A. D. & Trowsdale, J. The extended human leukocyte receptor complex: diverse ways of modulating immune responses. Immunol. Rev. 224, 98–123 (2008).
34. Mayeaud, L. LAIR and collagens in immune regulation. J. Biol. Chem. 280, 33101–33108 (2005).
35. Takai, T., Nakamura, A. & Endo, S. Role of PIR-B in autoimmune glomerulonephritis. J. Biomed. Biotechnol. 2011, 275302 (2011).
36. Atwal, J. K. et al. PIR-B is a functional receptor for myelin inhibitors of axonal regeneration. Science 322, 967–970 (2008).
37. Syken, J., Grandpre, T., Kanold, P. O. & Shatz, C. J. PIR-B restricts ocular-dominance plasticity in visual cortex. Science 313, 1795–1800 (2006).
38. Chen, R. J. et al. SHIP-2 heterozygous hematopoietic stem cells have deficient repopulating ability due to diminished self-renewal. Exp. Hematol. 34, 1229–1238 (2006).
39. Si, J. & Collins, S. J. Activated Ca2+ /calmodulin-dependent protein kinase II is a critical regulator of myeloid leukemia cell proliferation. Cancer Res. 68, 3733–3742 (2008).
METHODS

Mice. C57BL/6 and C57BL/6 CD45.1 mice, or NOG/SCID mice, were purchased from the University of Texas Southwestern Medical Center animal breeding core facility. The PIRBt mice were obtained from the MMRRC. The PIRB knockout mice were a gift from T. Takai. Mice were maintained at the University of Texas Southwestern Medical Center animal facility. All animal experiments were performed with the approval of the University of Texas Southwestern Committee on Animal Care.

Plasmids and proteins. Plasmid CMV–Kozak human Ang1 and ANGPTL1, 2, 3, 4, 6, and 7 with Flag tags at the C terminus were transfected into 293T cells using Lipofectamine 2000, and the conditioned medium at 48 h was collected and different ANGPTL proteins were adjusted to the same level for flow cytometry-based binding experiments. ANGPTL2–Flag was purified using M2 resin. Purified GST–ANGPTL5 was purchased from Abnova. Bacterially expressed Flag–ANGPTL2 and ANGPTL2–Flag were constructed in pET-28b (+) vectors, and GST–ANGPTL–Flag in pGEX vectors, and expressed and purified from bacteria.

MSCV–LILRB2–ires–GFP or control retrovirus-infected Ba3 cells, or CMV–driven LILRA–I–, LILRB–I, PIRB–I, or PIRB–I transgenic 293T cells collected at 48 h, or mononuclear human cord blood cells were incubated with Fc block and equal amounts of different Flag-tagged ANGPTLs at 4 °C for 60 min, followed by staining with anti-Flag–allophycocyanin (APC) and propidium iodide. Anti-LILRB2–phycoerythrin (PE) was used as indicated. Cells were analysed using either a FACScalibur or FACSaria instrument (Becton, Dickinson).

Antibodies and shRNAs. Flow cytometry antibodies anti-CD34–fluorescein isothiocyanate (FITC), anti-CD38–PE, anti-CD90–PE/Cy5.5, and biotinylated lineage antibodies and shRNAs.

Coimmunoprecipitation. ANGPTL2–Flag was purified using M2 resin. Purified GST–ANGPTL5 was purchased from Abnova. Bacterially expressed Flag–ANGPTL2 and ANGPTL2–Flag were constructed in pET-28b (+) vectors, and GST–ANGPTL–Flag in pGEX vectors, and expressed and purified from bacteria.

MSCV–LILRB2–ires–GFP or control retrovirus-infected Ba3 cells, or CMV–driven LILRA–I–, LILRB–I, PIRB–I, or PIRB–I transgenic 293T cells collected at 48 h, or mononuclear human cord blood cells were incubated with Fc block and equal amounts of different Flag-tagged ANGPTLs at 4 °C for 60 min, followed by staining with anti-Flag–allophycocyanin (APC) and propidium iodide. Anti-LILRB2–phycoerythrin (PE) was used as indicated. Cells were analysed using either a FACScalibur or FACSaria instrument (Becton, Dickinson).

Antibodies and shRNAs. Flow cytometry antibodies anti-CD34–fluorescein isothiocyanate (FITC), anti-CD38–PE, anti-CD90–PE/Cy5.5, and biotinylated lineage antibodies and shRNAs.

Coimmunoprecipitation. ANGPTL2–Flag was purified using M2 resin. Purified GST–ANGPTL5 was purchased from Abnova. Bacterially expressed Flag–ANGPTL2 and ANGPTL2–Flag were constructed in pET-28b (+) vectors, and GST–ANGPTL–Flag in pGEX vectors, and expressed and purified from bacteria.

MSCV–LILRB2–ires–GFP or control retrovirus-infected Ba3 cells, or CMV–driven LILRA–I–, LILRB–I, PIRB–I, or PIRB–I transgenic 293T cells collected at 48 h, or mononuclear human cord blood cells were incubated with Fc block and equal amounts of different Flag-tagged ANGPTLs at 4 °C for 60 min, followed by staining with anti-Flag–allophycocyanin (APC) and propidium iodide. Anti-LILRB2–phycoerythrin (PE) was used as indicated. Cells were analysed using either a FACScalibur or FACSaria instrument (Becton, Dickinson).

Antibodies and shRNAs. Flow cytometry antibodies anti-CD34–fluorescein isothiocyanate (FITC), anti-CD38–PE, anti-CD90–PE/Cy5.5, and biotinylated lineage antibodies and shRNAs.

Coimmunoprecipitation. ANGPTL2–Flag was purified using M2 resin. Purified GST–ANGPTL5 was purchased from Abnova. Bacterially expressed Flag–ANGPTL2 and ANGPTL2–Flag were constructed in pET-28b (+) vectors, and GST–ANGPTL–Flag in pGEX vectors, and expressed and purified from bacteria.

MSCV–LILRB2–ires–GFP or control retrovirus-infected Ba3 cells, or CMV–driven LILRA–I–, LILRB–I, PIRB–I, or PIRB–I transgenic 293T cells collected at 48 h, or mononuclear human cord blood cells were incubated with Fc block and equal amounts of different Flag-tagged ANGPTLs at 4 °C for 60 min, followed by staining with anti-Flag–allophycocyanin (APC) and propidium iodide. Anti-LILRB2–phycoerythrin (PE) was used as indicated. Cells were analysed using either a FACScalibur or FACSaria instrument (Becton, Dickinson).

Antibodies and shRNAs. Flow cytometry antibodies anti-CD34–fluorescein isothiocyanate (FITC), anti-CD38–PE, anti-CD90–PE/Cy5.5, and biotinylated lineage antibodies and shRNas.

Coimmunoprecipitation. ANGPTL2–Flag was purified using M2 resin. Purified GST–ANGPTL5 was purchased from Abnova. Bacterially expressed Flag–ANGPTL2 and ANGPTL2–Flag were constructed in pET-28b (+) vectors, and GST–ANGPTL–Flag in pGEX vectors, and expressed and purified from bacteria.

MSCV–LILRB2–ires–GFP or control retrovirus-infected Ba3 cells, or CMV–driven LILRA–I–, LILRB–I, PIRB–I, or PIRB–I transgenic 293T cells collected at 48 h, or mononuclear human cord blood cells were incubated with Fc block and equal amounts of different Flag-tagged ANGPTLs at 4 °C for 60 min, followed by staining with anti-Flag–allophycocyanin (APC) and propidium iodide. Anti-LILRB2–phycoerythrin (PE) was used as indicated. Cells were analysed using either a FACScalibur or FACSaria instrument (Becton, Dickinson).

Antibodies and shRNAs. Flow cytometry antibodies anti-CD34–fluorescein isothiocyanate (FITC), anti-CD38–PE, anti-CD90–PE/Cy5.5, and biotinylated lineage antibodies and shRNas.

Coimmunoprecipitation. ANGPTL2–Flag was purified using M2 resin. Purified GST–ANGPTL5 was purchased from Abnova. Bacterially expressed Flag–ANGPTL2 and ANGPTL2–Flag were constructed in pET-28b (+) vectors, and GST–ANGPTL–Flag in pGEX vectors, and expressed and purified from bacteria.

MSCV–LILRB2–ires–GFP or control retrovirus-infected Ba3 cells, or CMV–driven LILRA–I–, LILRB–I, PIRB–I, or PIRB–I transgenic 293T cells collected at 48 h, or mononuclear human cord blood cells were incubated with Fc block and equal amounts of different Flag-tagged ANGPTLs at 4 °C for 60 min, followed by staining with anti-Flag–allophycocyanin (APC) and propidium iodide. Anti-LILRB2–phycoerythrin (PE) was used as indicated. Cells were analysed using either a FACScalibur or FACSaria instrument (Becton, Dickinson).

Antibodies and shRNas. Flow cytometry antibodies anti-CD34–fluorescein isothiocyanate (FITC), anti-CD38–PE, anti-CD90–PE/Cy5.5, and biotinylated lineage antibodies and shRNas.

Coimmunoprecipitation. ANGPTL2–Flag was purified using M2 resin. Purified GST–ANGPTL5 was purchased from Abnova. Bacterially expressed Flag–ANGPTL2 and ANGPTL2–Flag were constructed in pET-28b (+) vectors, and GST–ANGPTL–Flag in pGEX vectors, and expressed and purified from bacteria.

MSCV–LILRB2–ires–GFP or control retrovirus-infected Ba3 cells, or CMV–driven LILRA–I–, LILRB–I, PIRB–I, or PIRB–I transgenic 293T cells collected at 48 h, or mononuclear human cord blood cells were incubated with Fc block and equal amounts of different Flag-tagged ANGPTLs at 4 °C for 60 min, followed by staining with anti-Flag–allophycocyanin (APC) and propidium iodide. Anti-LILRB2–phycoerythrin (PE) was used as indicated. Cells were analysed using either a FACScalibur or FACSaria instrument (Becton, Dickinson).
Competitor bone marrow cells, and the mixture injected intravenously through the retro-orbital route into each of a group of 6–9-week-old CD45.1 mice previously irradiated with a total dose of 10 Gy. To measure reconstitution of transplanted mice, peripheral blood was collected at the indicated times post-transplant and CD45.1 and CD45.2 cells in lymphoid and myeloid compartments were measured as we described9,10. The analyses of Mac-1, Kit, Gr-1, CD3 and B20 populations in AML blood or bone marrow were performed by using anti-Mac-1–APC, anti-Kit–PE, anti-Gr-1–PE, anti-CD3–APC and anti-B220–PE.

Uncultured or cultured progenies of human cells were pooled together and the indicated portions were injected intravenously through the retro-orbital route into sub-lethally irradiated (250 rad) 6–8-week-old NOD/SCID mice. 8 weeks after transplantation, bone marrow nucleated cells from transplanted animals were analysed by flow cytometry for the presence of human cells as we described1,10. c.f.u. assays. 2,000 YFP+ Mac-1+ Kit+ bone marrow cells from AML mice were plated in methylcellulose (M3534; Stem Cell Technologies) for c.f.u.–granulocyte macrophage assays, according to the manufacturer’s protocols and our previously published protocol34. After 7 days, 2,000 cells from three dishes initially plated were used for secondary replating.

Surface plasmon resonance. Bicore 2000 and CM5 chips were used to analyse binding of purified ANGPTLs to the LILRB2 ECD fused to Fc, using a method similar to that previously described35. Recombinant protein A (Pierce) was pre-immobilized in two flow cells (2,000 response units) using the amine-coupling kit from GE Healthcare. LILRB2–Fc was injected into one of the flow cells to be captured by the protein A to reach ~300 response units. GST–ANGPTL5 was injected over the immobilized LILRB2 in HBS–EP (GE Healthcare) containing 0.01 M HEPES (pH 7.4), 0.15 M NaCl and 0.005% polysorbate 20. Each binding sensorgram from the sample flow cell, containing a captured LILRB2–Fc, was corrected for the protein A-coupled cell control. After each injection of an antigen solution, which induced the binding reaction, and the dissociation period during which the running buffer was infused, the protein A surface was regenerated by the injection of the regeneration solution containing 10 mM Na3PO4 (pH 2.5) and 500 mM NaCl. All captured LILRB2–Fc, with and without ANGPTL5 bound, was completely removed, and another cycle begun. All measurements were performed at 25 °C with a flow rate of 30 μl min⁻¹.

GSEA. GSEA36 was performed using GSEA v2.0 software (http://www.broadinstitute.org/gsea/index.jsp) with 1,000 phenotype permutations, and normalized enrichment score and false-discovery rate q-value were calculated.

Statistics. A two-tailed Student’s t-test was performed to evaluate the significance between experimental groups, unless otherwise indicated. The survival rates of the two groups were analysed using a log-rank test.

30. Ujike, A. et al. Impaired dendritic cell maturation and increased T<sub>H2</sub> responses in PIR-B(2/2) mice. Nature Immunol. 3, 542–548 (2002).
31. Simsek, T. et al. The distinct metabolic profile of hematopoietic stem cells reflects their location in a hypoxic niche. Cell Stem Cell 7, 380–390 (2010).
32. Zhang, C. C., Krieg, S. & Shapiro, D. J. HMG-1 stimulates estrogen response element binding by estrogen receptor from stably transfected HeLa cells. Mol. Endocrinol. 13, 632–643 (1999).
33. Yan, M. et al. A previously unidentified alternatively spliced isoform of t(8;21) transcript promotes leukemogenesis. Nature Med. 12, 945–949 (2006).
34. Zhang, C. C., Steele, A. D., Lindquist, S. & Lodish, H. F. Prion protein is expressed on long-term repopulating hematopoietic stem cells and is important for their self-renewal. Proc. Natl Acad. Sci. USA 103, 2184–2189 (2006).
35. Luo, Y., Lu, Z., Raso, S. W., Entrican, C. & Tangarone, B. Dimers and multimers of monoclonal IgG1 exhibit higher in vitro binding affinities to Fcγ receptors. MAbs 1, 491–504 (2009).
36. Subramanian, A. et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl Acad. Sci. USA 102, 15545–15550 (2005).
37. Zuber, J. et al. RNAi screen identifies Brd4 as a therapeutic target in acute myeloid leukaemia. Nature 478, 524–528 (2011).
CORRIGENDUM

do:10.1038/nature11268

Corrigendum: Inhibitory receptors bind ANGPTLs and support blood stem cells and leukaemia development

Junke Zheng, Masato Umikawa, Changhao Cui, Jiyuan Li, Xiaoli Chen, Chaozheng Zhang, HoangDinh Huynh, Xunlei Kang, Robert Silvany, Xuan Wan, Jingxiao Ye, Alberto Puig Cantó, Shu-Hsia Chen, Huan-You Wang, E. Sally Ward & Cheng Cheng Zhang

Nature 485, 656–660 (2012); doi:10.1038/nature11095

The surname of author HoangDinh Huynh was inadvertently misspelled as Hyunh. This has been corrected online in the PDF and HTML of the original paper.