Mesenchymal PGD$_2$ activates an ILC2-Treg axis to promote proliferation of normal and malignant HSPCs

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Abstract

Cyclooxygenase (COX)-dependent production of prostaglandins (PGs) is known to play important roles in tumorigenesis. PGD$_2$ has recently emerged as a key regulator of tumor- and inflammation-associated functions. Here we show that mesenchymal stromal cells (MSCs) from patients with acute myeloid leukemia (AML) or normal MSCs overexpressing COX2 promote proliferation of co-cultured hematopoietic stem and progenitor cells (HSPCs), which can be prevented by treatment with COX2 knockdown or TM30089, a specific antagonist of the PGD$_2$ receptor CRTH2. Mechanistically, we demonstrate that PGD$_2$-CRTH2 signaling acts directly on type 2 innate lymphoid cells (ILC2s), potentiating their expansion and driving them to produce Interleukin-5 (IL-5) and IL-13. Furthermore, IL-5 but not IL-13 expands CD4$^+$CD25$^+$IL5Rα^+ T regulatory cells (Tregs) and promotes HSPC proliferation. Disruption of the PGD$_2$-activated ILC2-Treg axis by specifically blocking the PGD$_2$ receptor CRTH2 or IL-5 impedes proliferation of normal and malignant HSPCs. Conversely, co-transfer of CD4$^+$CD25$^+$IL5Rα^+ Tregs promotes malignant HSPC proliferation and accelerates leukemia development in xenotransplanted mice. Collectively, these results indicate that the mesenchymal source of PGD$_2$ promotes proliferation of normal and malignant HSPCs through activation of the ILC2-Treg axis. These findings also suggest that this novel PGD$_2$-activated ILC2-Treg axis may be a valuable therapeutic target for cancer and inflammation-associated diseases.

Introduction

Prostaglandins (PGs) are lipid compounds of eicosanoid family, which play major roles in inflammation and immune responses (1). Synthesized by the pro-inflammatory cyclooxygenases, COX1 and COX2, through oxidation of the derivative arachidonic acid, major prostaglandins include PGD_2, PGE_2, PGH_2 and PGI_2 (2). We previously reported that mesenchymal stromal cells (MSCs) from patients with acute myeloid leukemia (AML) overexpressed COX2 and secreted high levels of PGs including PGD_2 (3). PGD_2 is also produced by activated mast cells, macrophages, and T helper 2 (Th2) cells. The biological actions of PGD_2 are mediated through two G-protein-coupled receptors, prostanoid DP receptor (DP) and chemoattractant receptor homologous molecules expressed on Th2 cells (CRTH2, 4–9). PGD_2 signaling through DP and CRTH2 mediates different and often opposite effects in many cell types of the immune system (10). Within the immune system, DP activation affects the maturation process and migratory ability of human and mouse dendritic cells (DCs, 11–13). On the other hand, CRTH2 was identified in human on type 2 polarized lymphocytes (14), basophils (4), eosinophils and monocytes (13). PGD_2 has recently emerged as a key regulator of tumor- and inflammation-associated functions (15–18). However, little is known about the role of PGD_2 in normal and malignant hematopoiesis.

Innate lymphoid cells (ILCs) represent a novel family of hematopoietic effectors that play crucial roles in the early immune response through cytokine and chemokine secretion (19). ILCs are divided into three functional groups: ILC1, ILC2 and ILC3 that have parallels with the T helper (Th) subsets of Th1, Th2 and Th17 (20). ILC2s, much in common with Th2 cells, play a major role in protection from disease on the one hand, and drive autoimmune disease on the other hand (21). It has been shown that PGD_2 activates ILC2 through CRTH2 expressed on Th2 cells (22), and leads to production of the classical type 2 cytokines, thus promoting type 2 immunity (23). Although dysregulation or chronic activation of ILC2 has been reported in pathologic conditions, such as allergy, atopic dermatitis and nasal polyposis (21, 24), the role of ILC2s in regulating hematopoiesis remains elusive.

Acute myeloid leukemia (AML) is the most common acute leukemia in adults (25). Immune system impairment has been reported in AML patients (26, 27). These defects influence the function of regulatory T cells (Tregs), which suppress the proliferation and function of Th cells (26, 28). Tregs have been recognized as a contributing factor in leukemogenesis, and may be recruited and exploited by leukemic cells to evade immune surveillance. Indeed, patients with AML show abnormally high level of Tregs within their peripheral blood (PB) and bone marrow (BM) compared with healthy donors (29), which correlates with the poor treatment outcome of AML patients (30). Mouse studies also show that Tregs accumulate in leukemic tissues and impede the proliferative and cytolytic capacity of adoptively transferred anti-AML reactive cytotoxic T lymphocytes (CTLs). This suppressive effect can be reversed when Tregs and other T lymphocytes are removed from the microenvironment *in vitro*, leading to augmented immune responses to AML (31). The mechanistic link between Tregs and malignant hematopoiesis is less understood.

In the present study, we have investigated the role of MSC-derived PGD_2 in promoting the proliferation of normal and malignant hematopoietic stem and progenitor cells (HSPCs). Mechanistically, we found that PGD_2-CRTH$_2$ signaling acts directly on ILC2s, potentiating their expansion and driving them to produce IL-5, which in turn expands $CD4^+CD25^+IL$-5Rα^+ Tregs and promotes HSPC proliferation. These findings identify a novel PGD_2-acitvated ILC2-Treg axis that may be a valuable therapeutic target for cancer and inflammation-associated diseases.

Materials and Methods

Human bone marrow stromal cell (hBMSC) culture and treatment

All studies with human material were approved by the institutional review board and ethics committee of the West Virginia University (WVU). Written informed consent was obtained from all subjects. Characteristics of the AML samples used in this study was summarized in Table S1. Human MSC culture protocol was adapted and modified from previously described method (3). Briefly, cell cultures were maintained at 37˚C, in 5% CO2 and 98% humidity in normal growth medium consisting of Dulbecco's modified eagle medium (DMEM-low glucose, with GlutaMAX; ThermoFisher Scientific, cat # 10567022, Reinach, Switzerland), supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St Louis, MO), penicillin/Streptomycin (50 units/ml; 50 μg/m; Sigma-Aldrich, Cat # F9665, St Louis, MO). All MSCs were used at passage 3, which displayed characteristic MSC surface phenotype ($CD45^-HLA$-DR$^-CD105^+CD73^+CD90^+$ $CD44^+CD146^+$, ref 3; BD Pharmingen, Cat #562245, San Jose, CA).

BW8668C was purchased from Cayman Chemical (Ann Arbor, MI). TM30089 was supplied by ChemieTek (cat # CT-AT002, Indianapolis, IN). Both of the antagonists were used at 1 μM concentration for *ex vivo* co-culture.

Bone marrow hematopoietic stem progenitor cell (HSPC)-MSC co-culture

1,000 bone marrow $CD34^+$ cells from healthy donor or patients with acute myeloid leukemia (AML) were seeded on 70% confluent MSCs derived from different sources and culture in serum-free medium containing 100 ng/ml each of SCF, Flt3L, TPO and G-CSF (Peprotech, Cat # 30007, 30019, 30018, 30023, Rocky Hill, NJ). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO2. The co-cultures were incubated for 4–10 days, at which time-periods the progeny of the co-cultured HSPCs was harvested and analyzed (3).

Results

AML MSCs overexpress COX2 and promote normal HSPC proliferation

We previously reported that mesenchymal stromal cells (MSCs) from patients with acute myeloid leukemia (AML) overexpress COX2 and secreted high levels of prostaglandins (PGs) including PGD_2 (3). Since little is known about the role of PGD_2 in normal and malignant hematopoiesis, we prioritized this mesenchymal source of PG for further investigation. To determine the effects of MSCs-derived PGD_2 on HSPC proliferation, we

employed two approaches: 1) knockdown (KD) of *COX2* in AML MSCs; and 2) overexpression of *COX2* in healthy donor (HD) MSCs. First, we reduced COX2 expression in the AML-MSCs by shRNA KD (Fig. S1A). Enzyme-linked immunosorbent assay (ELISA) validated decreased production of PGD_2 in *COX2*-KD AML MSCs (Fig. S1B). Importantly, we found that co-culture of normal HSPCs with AML MSCs leads to 5–10 fold expansion of HSC-enriched $CD34^+CD38^-$ cells on days 4–10 in co-culture (Fig. 1A). Knocking down of *COX2* in AML MSCs significantly reduced the expansion of $CD34^+CD38^-$ cells compared to Scramble controls (Fig. 1A). Consistently, *COX2*-KD in AML MSCs markedly reduced the proliferation of the co-cultured $CD34^+CD38^-$ cells, as measured by BrdU incorporation assay (Fig. 1B). Conversely, ectopic overexpression of COX2 by viral gene transfer in HD MSCs increased secretion of PGD_2 (Fig. S1C–E) and significantly expanded normal $CD34^+CD38^-$ cells in co-culture (Fig. 1C), which was correlated with increased proliferation of the co-cultured $CD34^+CD38^-$ cells (Fig. 1D). These data indicate that overexpression of COX2 and PGD_2 in MSCs correlates with increased proliferation of normal HSPCs.

CRTH2 mediates the effect of mesenchymal PGD_2 in HSPC expansion

The biological effects of PGD_2 are known to be mediated by two distinct receptors: D-prostanoid (DP), and chemoattractant receptor homologous molecules expressed on Th2 cells (CRTH2, 4–9). To determine whether the effect of overexpressed COX2 by AML MSCs in the promotion of normal HSPC proliferation was mediated by mesenchymal PGD_2, we first attempted to identify the receptor that mediates the effect of AML MSC-derived PGD_2. To this end, we employed both genetic and pharmacological approaches: *COX2*-KD to deplete PGD_2 in AML MSCs; utility of the inhibitors for the two PGD_2 receptors: BW8668C, a selective DP antagonist (32), and TM30089, a specific CRTH2 antagonist (23, 33). In this setting, if mesenchymal COX2-derived PGD_2 promotes HSPC expansion, inhibition of the PGD_2-producing enzyme by *COX2*-KD or the PGD_2 receptors by BW8668C or TM30089 would reduce expansion of the co-cultured HSPCs. We found that *COX2*-KD of AML MSCs effectively reduced the expansion of co-cultured normal $CD34^+CD38^-$ HSPCs (Fig. 2A, B). The DP antagonist BW868C failed to prevent HSPC expansion after 4 days co-culture with AML MSCs (Fig. 2A). However, treatment of AML MSCs with the CRTH2 antagonist TM30089 exhibited strong suppression of the co-cultured normal $CD34^+CD38^-$ HSPCs (Fig. 2B). Thus, CRTH2 mediates the effect of mesenchymal PGD_2 in HSPC expansion.

Since the cognate PGD_2-CRTH2 ligation activates ILC2s and prompts them to secret type 2 cytokines such as IL-4, IL-5 and IL-13 (23, 34), we next investigated the effect of mesenchymal PGD_2 on ILC2 maintenance. Because ILC2s are best defined by the expression of interleukin (IL)-7 receptor alpha chain (CD127) and CRTH2 (Fig. S2A), we performed a time-course analysis of the frequency of $CD127^+CRTH2^+ILC2s$ in the progeny of BM $CD34^+$ cells co-cultured on AML MSCs, and observed a peak of ILC2 expansion on day 4 (Fig. S2B). We first compared the frequencies of $CD127^+CRTH2^+ILC2s$ derived from normal $CD34^+$ HSPCs co-cultured on HD or AML MSCs. We observed a significantly increased frequency of $CD127^+CRTH2^+ILC2s$ in the progeny of the $CD34^+$ cells after 4 days of co-culture on AML MSCs compared to those on HD MSCs (Fig. 2C). We then

analyzed the frequency of $CD127^+CRTH2^+ILC2s$ derived from normal $CD34^+$ HSPCs co-cultured on AML MSCs that had subjected to *COX2*-KD. We found that *COX2*-KD effectively reduced the expansion of these $CD127^+CRTH2^+ILC2s$ compared to the Scramble control virus (Fig. 2D). Consistently, analysis of the expression of the two PGD_2 receptors, CRTH2 and DP, on Lin^-c-kit^-CD127^+ ILC cells showed that that *COX2*-KD affected the expression of CRTH2 but not that of DP (Fig. 2E). Together, these results identify CRTH2 as the receptor that mediates the effect of mesenchymal PGD_2 and suggest that mesenchymal PGD_2 promotes HSPC proliferation through activating ILC2s.

Activation of the mesenchymal PGD_2-CRTH2/ILC2 signaling axis overproduces IL-5, which promotes HSPC expansion

It is known that PGD_2-activated ILC2s preferentially elicits the production of type-2 effector cytokines IL-4, IL-5 and IL-13 (9). We therefore attempted to further identify the effector cytokine(s) of PGD_2-CRTH2 signaling in promoting HSPC proliferation. To this end, we sorted $CD127^+CRTH2^+$ ILC2s (Fig. S2C) for co-culture with HD or AML MSCs and determined the expression of the effector cytokines at the levels of mRNA and protein secreted to the culture supernatants. We found that $CD127^+CRTH2^+$ ILC2s co-cultured on AML MSCs expressed significantly higher levels of IL-5 and IL-13, but not IL-4, as measured by qPCR (Fig. S2D) and ELISA (Fig. S2E), than those co-cultured on HD MSCs. *COX2*-KD, or treatment of AML MSCs with the CRTH2 antagonist TM30089 significantly reduced the levels of IL-5 and IL-13 in co-cultured $CD127^+CRTH2^+$ ILC2s (Fig. 3A, B). These results indicate that mesenchymal PGD_2 activates the CRTH2/ILC2 signaling to increase the production of IL-5 and IL-13 effector cytokines.

We next determined whether $CD127^+CRTH2^+$ ILC2s was sufficient to promote HSPC proliferation by co-culturing FACS-sorted $CD127^+CRTH2^+$ ILC2s (Fig. S2C) with normal BM $CD34^+$ cells followed by assessing the expansion of $CD34^+CD38^-$ HSPCs. Surprisingly, we observed that in contrast to AML MSCs, $CD127^+CRTH2^+ILC2s$ alone failed to promote the expansion of $CD34^+CD38^-$ cells (Fig. 3C). This suggests that $CD127^+CRTH2^+ILC2s$ did not act directly on the co-cultured HSPCs and prompted us to further search for the downstream effector(s). Because we observed increased secretion of IL-5 and IL-13 by mesenchymal PGD_2-activated ILC2s, we attempted to determine whether IL-5 or/and IL-13 acted as effector cytokines in promoting HSPC proliferation. The results showed that addition of anti-IL-5, but not anti-IL-13, neutralizing antibodies completely abolished the effect of AML MSCs on the expansion of co-cultured HSPCs (Fig. 3D). Collectively, these results indicate that mesenchymal PGD_2 promotes HSPC expansion through the effector cytokine IL-5 secreted by activated ILC2s.

ILC2s-produced IL-5 expands $CD4^+CD25^+IL$-$5R\alpha^+$ Tregs, which promote HSPC expansion

Since IL-5 promotes generation of antigen-specific $CD4^+CD25^+$ T regulatory cells (Tregs, 35, 36), and since emerging evidence suggests that Tregs function as an important component of the supporting niche for HSPCs (37, 38), we wondered if the ILC2s/IL-5-Treg signaling axis was the underlying mechanism of mesenchymal PGD_2-mediated HSC proliferation. We first compared the frequencies of $CD4^+CD25^+$ Tregs in the co-culture of normal $CD34^+$ HSPCs on HD and AML MSCs. We found that $CD34^+$ HSPCs co-cultured

on AML MSCs produced significantly higher frequencies of CD4$^+$CD25$^+$ Tregs than those co-cultured on HD MSCs (Fig. 4A). We then determined Treg frequencies in the co-culture of normal CD34$^+$ HSPCs on AML MSCs that had subjected to treatment with *COX2*-KD or the CRTH2 antagonist TM30089 or the anti-IL-5 neutralizing antibodies. In this setting, if mesenchymal COX2-derived PGD$_2$ promoted the generation of CD4$^+$CD25$^+$ Tregs, targeting the PGD$_2$-producing enzyme by *COX2*-KD, or the PGD$_2$ receptor CRTH2 by TM30089, or IL-5 blockage by the anti-IL-5 neutralizing antibodies, would reduce the frequency of CD4$^+$CD25$^+$ Tregs differentiated from the co-cultured HSPCs. Indeed, treatment of AML MSCs with *COX2*-KD, or the CRTH2 antagonist TM30089, or the anti-IL-5 neutralizing antibodies, significantly reduced CD4$^+$CD25$^+$ Tregs derived from the co-cultured CD34$^+$ HSPCs (Fig. 4B). Furthermore, AML MSCs-expanded CD4$^+$CD25$^+$ Tregs expressed high levels of IL5Rα (Fig. 4D), which was effectively reduced by *COX2*-KD, or TM30089 or IL-5 blockade (Fig. 4C). These results indicate that IL-5 produced by mesenchymal PGD$_2$-activated ILC2s expands CD4$^+$CD25$^+$IL-5Rα^+ Tregs.

Because one functional hallmark of Tregs is the suppression of T effector cell (Teff) proliferation (39), we performed the CFSE retention assay to determine the capacity of ILC2s-expanded CD4$^+$CD25$^+$IL-5Rα^+ Tregs in the suppression of Teff proliferation. We purified CD4$^+$CD25$^+$IL-5Rα^+ and CD4$^+$CD25$^+$IL-5Rα^- Tregs from CD34$^+$ HSPCs-AML MSC co-culture (Fig. S3A), and cultured these two subsets of Tregs with CD4$^+$CD25$^-$ Teffs in the presence of CFSE dye. We found that CD4$^+$CD25$^+$IL5Rα^+ Treg did not show higher potential in suppressing the proliferation of CD4$^+$CD25$^-$ Teffs than their CD4$^+$CD25$^+$IL5Rα^- counterparts (Fig. S3B). However, CD4$^+$CD25$^+$IL5Rα^+ Tregs exhibited significantly higher potential in promoting the expansion of co-cultured CD34$^+$CD38$^-$ HSPCs than CD4$^+$CD25$^+$IL5Rα^- Tregs did (Fig. 4E). Taken together, these results identified IL-5 as the effector cytokine produced by mesenchymal PGD$_2$-activated ILC2s, which expands CD4$^+$CD25$^+$IL5Rα^+ Tregs and promotes HSPC expansion.

Mesenchymal PGD$_2$-expanded CD4$^+$CD25$^+$ Tregs produce IL-10, which promotes HSPC expansion

IL-10 produced by Tregs plays a key role in immune suppression and limiting inflammation (37, 40–42). IL-10 deficiency induces anemia and hematopoietic anomalies in mice (43). Therefore, we hypothesized that IL-10 produced by IL-5-expanded Tregs might be responsible for the mesenchymal PGD$_2$-mediated HSPC proliferation, as the CD4$^+$CD25$^+$ Tregs derived from HSPCs co-cultured on AML MSCs produced significantly higher levels of IL-10 than those from HSPCs co-cultured on HD MSCs (Fig. S4). We next determined the intra-cellular levels of IL-10 in CD4$^+$CD25$^+$ Tregs-derived from the normal CD34$^+$ HSPCs co-cultured on AML MSCs that had subjected to treatment with *COX2*-KD, or the CRTH2 antagonist TM30089, or the anti-IL-5 neutralizing antibodies. We found that treatment of AML MSCs with *COX2*-KD, or the CRTH2 antagonist TM30089, or the anti-IL-5 neutralizing antibodies, significantly reduced the intra-cellular levels of IL-10 in CD4$^+$CD25$^+$ Tregs derived from the co-cultured CD34$^+$ HSPCs (Fig. 5A). Consistently, qPCR analysis of *IL-10* mRNA transcript and ELISA measurement of the IL-10 proteins secreted into the culture supernatants showed that *COX2*-KD, TM30089 or IL-5 blockade significantly reduced the levels of *IL-10* mRNA (Fig. 5B) and protein (Fig. 5C). More

importantly, IL-10 neutralization showed as effective as *COX2*-KD or TM30089 in impeding the effect of AML MSCs on promoting HSPC expansion (Fig. 5D). Thus, it appears that IL-10 produced by IL-5-expanded Tregs is the ultimate promotor for the mesenchymal PGD_2-mediated HSPC expansion.

Mesenchymal PGD_2-activated ILC2-Treg axis promotes proliferation of malignant HSPCs

We next investigated the role of the mesenchymal PGD_2-activated ILC2-Treg axis in malignant hematopoiesis. We co-cultured $CD34^+$ cells from AML patients on AML MSCs that had subjected to treatment with *COX2*-KD, the CRTH2 antagonist TM30089, or the anti-IL-5 neutralizing antibodies. We observed a significant reduction in the expansion of leukemic stem cell (LSC)-enriched $CD34^+CD38^-$ cells derived from the $CD34^+$ leukemic progenitor cells co-cultured on the AML MSCs treated with *COX2*-KD, TM30089, or the anti-IL-5 neutralizing antibodies (Fig. 6A). We also performed co-culture of the $CD34^+$ leukemic progenitor cells on HD MSCs that overexpressed COX2 and treated with TM30089, or the anti-IL-5 neutralizing antibodies. In this setting, if overexpression of COX2 in HD MSCs promoted the expansion of LSC-enriched $CD34^+CD38^-$ cells through the PGD_2-activated ILC2-Treg axis, targeting the PGD_2 receptor CRTH2 by TM30089, or IL-5 blockade by the anti-IL-5 neutralizing antibodies, would limit the expansion of leukemic $CD34^+CD38^-$ cells. Indeed, overexpression of *COX2* in HD MSCs promoted the expansion of AML $CD34^+CD38^-$ cells, which was completely abolished by TM30089 or anti-IL-5 treatment (Fig. 6B).

To extend these *in vitro* findings, we performed BM transplantation to investigate the *in vivo* role of the mesenchymal PGD_2-activated ILC2-Treg axis in normal and malignant hematopoiesis, by co-transplanting normal or AML $CD34^+$ progenitor cells with $Lin^-CD127^+CRTH2^+$ ILC2s or $CD4^+CD25^+IL5R\alpha^+$ Tregs, sorted from the progeny of the normal $CD34^+$ HSPCs co-cultured on AML MSCs, into sublethally irradiated NSGS mice (44, 45). We also treated the recipients co-transplanted with HSPCs and $CD4^+CD25^+IL5R\alpha^+$ Tregs with antibodies targeting the IL-10 receptor (IL-10R) or IgG control. Analysis of human engraftment six weeks post-transplant showed that $CD4^+CD25^+IL5R\alpha^+$ Tregs, but not $Lin^-CD127^+CRTH2^+$ ILC2s, promoted the proliferation of normal HSPCs *in vivo,* as evidenced by a significant increase in both total and BrdU-positive donor-derived $CD34^+CD38^-$ HSPCs in the recipients co-transplanted with $CD4^+CD25^+IL5R\alpha^+$ Tregs compared to those recipients transplanted with normal $CD34^+$ cells alone or co-transplanted with $Lin^-CD127^+CRTH2^+$ ILC2s (Fig. 6C, 6D). The blockade of IL-10 signaling by anti-IL-10R antibodies almost completely abolished the effect of $CD4^+CD25^+IL5R\alpha^+$ Tregs in promoting the proliferation of normal HSPCs (Fig. 6C, 6D). In addition, *in vivo* depletion of $CD4^+CD25^+IL5R\alpha^+$ Tregs using anti-CD25 and anti-IL5Rα antibodies significantly limited the proliferation of normal HSPCs in the secondary transplanted recipients (Fig. 6E), confirming that $CD4^+CD25^+IL5R\alpha^+$ Tregs promote proliferation of normal HSPCs.

Finally, we co-transplanted humanized NSGS mice with AML $CD34^+$ cells and $Lin^-CD127^+CRTH2^+$ ILC2s or $CD4^+CD25^+IL5R\alpha^+$ Tregs, and found that similar to the co-transplant experiments with normal HSPCs, $CD4^+CD25^+IL5R\alpha^+$ Tregs significantly increased both total human ($hCD45^+$) engraftment and LSC-enriched $CD34^+CD38^-$ cells 6

weeks post-transplant in the recipients co-transplanted with CD4$^+$CD25$^+$IL5Rα^+ Tregs compared to the recipients transplanted with leukemic CD34$^+$ cells alone or co-transplanted with Lin$^-$CD127$^+$CRTH2$^+$ ILC2s (Fig. 7A). Furthermore, a marked increase in BrdU incorporation was observed in donor-derived CD34$^+$CD38$^-$ cells in the recipients co-transplanted with CD4$^+$CD25$^+$IL5Rα^+ Tregs compared to other three groups (Fig. 7B). Additionally, it appeared that the AML donor cells underwent myeloid expansion in the NSGS recipients (Fig. 7A). Blocking IL-10 signaling by anti-IL-10R antibodies effectively eliminated the proliferation-promoting effect of CD4$^+$CD25$^+$IL5Rα^+ Tregs (Fig. 7A, 7B). Moreover, we observed that all recipient mice developed leukemia within four months after transplantation (Fig. 7C). CD4$^+$CD25$^+$IL5Rα^+ Tregs accelerated leukemia development; whereas anti-IL-10R blockade ameliorated the leukemia-promoting effect of CD4$^+$CD25$^+$IL5Rα^+ Tregs (Fig. 7C). We noted that anti-IL-10R blockade did not have effect on either the proliferation of leukemic HSPCs or leukemia development in the recipients transplanted with the AML CD34$^+$ cell alone (Fig. 7A–C). Consistently, further characterization of the leukemic mice showed the opposite effects of CD4$^+$CD25$^+$IL5Rα^+ Tregs and anti-IL-10R blockade on the proliferation of leukemic cells (Fig. S5A) and leukemic burden, as measured by WBC counts (Fig. S5B), and infiltration of leukemic blasts in the PB, BM and spleen (Fig. S5C). We also observed that both CD4$^+$CD25$^+$IL5Rα^+ Tregs and IL-10 increased progressively in co-transplanted mice of AML CD34$^+$ cells during six weeks post-transplant (Fig. S5D–E). Furthermore, secondary transplantation experiments revealed that *in vivo* depletion of CD4$^+$CD25$^+$IL5Rα^+ Tregs significantly limited the proliferation the leukemic HSPCs in the secondary recipients (Fig. 7D), further demonstrating that CD4$^+$CD25$^+$IL5Rα^+ Tregs promote proliferation of malignant HSPCs. In line with these observations, co-transplantation of primary leukemic Lin$^-$CD127$^+$CRTH2$^+$ILC2 or CD4$^+$CD25$^+$IL5Rα^+ Tregs sorted from leukemic mice showed that leukemic CD4$^+$CD25$^+$IL-5Rα^+ Tregs and to a lesser degree, Lin$^-$CD127$^+$ CRTH2$^+$ ILC2s promote the proliferation of normal and malignant HSPCs in the transplanted recipient mice (Fig. 7E and 7F). Together, the data indicate that the PGD$_2$-activated ILC2-Treg axis promotes proliferation of normal and malignant HSPCs in NSGS recipients.

Discussion

Mesenchymal prostaglandins (PGs) are important components of the stromal secretome (46), and play major roles in inflammation and immune response (47). We previously demonstrated that MSCs from patients with AML overexpress COX2 enzyme and secrete high levels of PGs, including PGD$_2$ (3), a major arachidonic acid metabolite also produced by activated mast cells, macrophages and Th2 cells (7, 8). Here we have investigated the role of mesenchymal PGD$_2$ in the regulation of normal and malignant hematopoiesis and demonstrate that this unique source of PGD$_2$ promotes proliferation of normal and malignant HSPCs through activation of a novel ILC2-Treg axis. Mechanistically, we employed both genetic and pharmacological approaches to delineate three critical and sequential events leading to the activation of the ILC2-Treg axis by mesenchymal PGD$_2$ (Fig. 8): 1) Mesenchymal COX2-derived PGD$_2$ engages the receptor CRTH2 on ILC2s and promotes ILC2 expansion; 2) Mesenchymal PGD$_2$-activated ILC2s overproduces type II effector cytokine IL-5, which expands CD4$^+$CD25$^+$IL5Rα^+ Tregs; 3) Mesenchymal PGD$_2$-

expanded CD4$^+$CD25$^+$IL5Rα^+ Tregs produce IL-10, which promotes co-cultured HSPC proliferation. Functionally, we provided *in vivo* evidence that mesenchymal PGD$_2$-activated ILC2-Treg axis promotes proliferation of both normal and malignant HSPCs in xenotransplanted recipients. Our finding suggests that this novel ILC2-Treg axis over-activated by mesenchymal PGD$_2$ may be a valuable therapeutic target for cancer and inflammation-associated diseases.

One interesting finding the present study is our observation that AML MSC-derived PGD$_2$ promotes HSPC proliferation through CRTH2 but not the DP signaling pathway. The biological actions of PGD$_2$ are mediated through two G-protein-coupled receptors, DP and CRTH2 (4–9). It is known that the CRTH2 receptor is involved in induction of migration and activation of Th2 lymphocytes, eosinophils, and basophils; upregulation of adhesion molecules; and promotion of pro-inflammatory Th2-type cytokines (IL-4, 5, 12), whereas the DP receptor is associated with relaxation of smooth muscles, vasodilation, inhibition of cell migration, and apoptosis of eosinophils (48). Recently, several studies have shown that hematopoietic source of PGD$_2$ specifically engages CRTH2 on ILC2s, which in turn mediate immune response in a variety of conditions such as allergic diseases and acute promyelocytic leukemia (9, 22, 23, 37, 49, 50). Our observation that mesenchymal PGD$_2$ also engages and activates ILC2s through CRTH2, thus adding another facet to the PGD$_2$-CRTH2/ILC2 signaling.

Our present studies show that COX2-derived PGD$_2$ activates ILC2s, which subsequently expands Tregs. We also demonstrate that CD4$^+$CD25$^+$IL5Rα^+ Tregs have higher potential in promoting co-cultured BM CD34$^+$ HSPC proliferation than the CD4$^+$CD25$^+$IL5Rα^- Tregs (Fig. 4D). One well-established function of Tregs is the suppression of Teff proliferation (39). Surprisingly, we observed no greater capacity of suppression of Teff proliferation by the ILC2s-expanded CD4$^+$CD25$^+$IL-5Rα^+ Tregs than the CD4$^+$CD25$^+$IL5Rα^- control subset. Tregs have also been implicated as an important contributing factor to be recruited and exploited by leukemic cells to evade immune surveillance and allow malignant hematopoietic cells to survive and proliferate (26, 27). On the other hand, Tregs have recently been characterized as key regulators of non-immunological processes in various tissues (51–55). In the bone marrow, it has been reported that Tregs function as a component of the bone marrow microenvironment (BM niche), providing allogeneic HSPCs with immune privilege and regulating HSC quiescence and engraftment (37, 38). It is in this context, our findings add another layer to the current understanding of BM microenvironment and the role of Tregs in normal and malignant hematopoiesis.

Previous studies have demonstrated that altered mesenchymal niche cells impede generation of normal hematopoietic progenitor cells in leukemic bone marrow (56, 57). In the present study, we show that mesenchymal COX2-derived PGD$_2$ activates an ILC2-Treg axis to produce IL-10, which subsequently promotes normal and malignant HSPC proliferation. IL-10 produced by Tregs is required for suppression of Th17 cell-mediated inflammation (41, 42). In fact, IL-10 deficiency impairs Treg-derived neuropilin-1 functions and promotes Th1 and Th17 immunity (58). The role of Treg-derived IL-10 in HSPC maintenance is less understood. A previous report identified a novel role of IL-10 in regulating HSC self-renewal (59). It is known that Tregs are a prime source of IL-10 (40, 60), and a recent study

has implicated Treg-derived IL-10 to be crucial for maintaining HSC quiescence (39). More recently, it has been reported that CD150^{high} BM Tregs maintain HSC quiescence and immune privilege via adenosine (47). Although it is unclear for the mechanism by which mesenchymal PGD$_2$-activates Tregs and Treg-derived IL-10 promotes HSPC proliferation, our studies, together with these previous studies, suggest a critical role of Tregs and Treg-derived IL-10 in normal and malignant hematopoiesis.

In summary, the current study demonstrates that Mesenchymal COX2-derived PGD$_2$ activates an ILC2-Treg axis to promote proliferation of normal and malignant HSPCs. These findings identify a novel PGD$_2$-acitvated ILC2-Treg axis that may be a valuable therapeutic target for cancer and inflammation-associated diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

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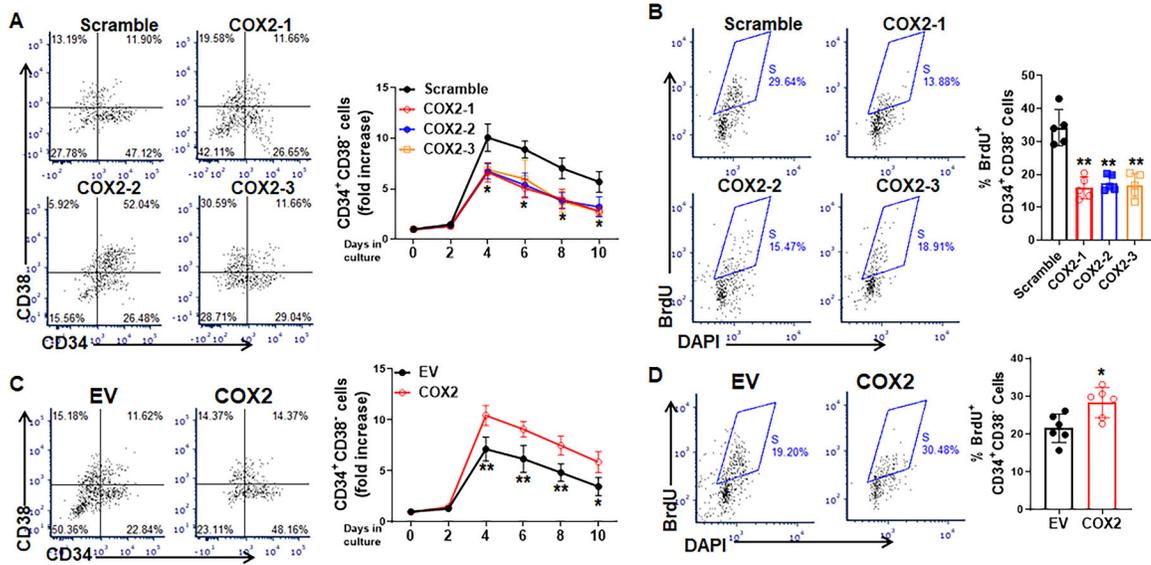
Fig 1. Mesenchymal COX2 promote proliferation of co-cultured HSPCs.
(A) Knocking down of *COX2* in AML MSCs suppresses HSPC expansion. 1,000 BM CD34$^+$ cells from healthy donor (HD) were co-cultured with MSCs derived from AML patients (AML MSCs) transduced with scramble shRNA or shRNA targeting *COX2* for 10 days. CD34$^+$CD38$^-$ cells derived from the co-cultured CD34$^+$ HSPCs were analyzed by flow cytometry at the indicated time points. Representative flow plots on day 4 (Left) and quantification (Right) are shown. HSPC expansion is expressed as fold increase in absolute numbers relative to pre-culture (day 0) levels. Results are means ± SD of three independent experiments. (B) Knocking down of *COX2* in AML MSCs reduces HSPC proliferation. Cells described in (A) were subjected to BrdU incorporation assay. Representative plots of BrdU-positive CD34$^+$CD38$^-$ cells (Left) and quantification (Right) were shown. Results are means ± SD of three independent experiments. (C) Ectopic over-expression of COX2 in HD MSCs promotes HSPC expansion. 1,000 BM CD34$^+$ cells from healthy donor (HD) were co-cultured with MSCs derived from HD (HD MSCs) transduced with vector expressing empty vector (EV)- or *COX2* for 10 days. CD34$^+$CD38$^-$ cells derived from the co-cultured CD34$^+$ HSPCs were analyzed by flow cytometry at the indicated time points. Representative flow plots on day 4 (Left) and quantification (Right) are shown. Results are means ± SD of three independent experiments. (D) Overexpression COX2 in HD MSCs promotes HSPC proliferation. Cells described in (C) were subjected to BrdU incorporation assay. Representative flow plot of BrdU-positive CD34$^+$CD38$^-$ cells (Left) and quantifications (Right) were shown. Results are means ± SD of three independent experiments (n=8 for each group).
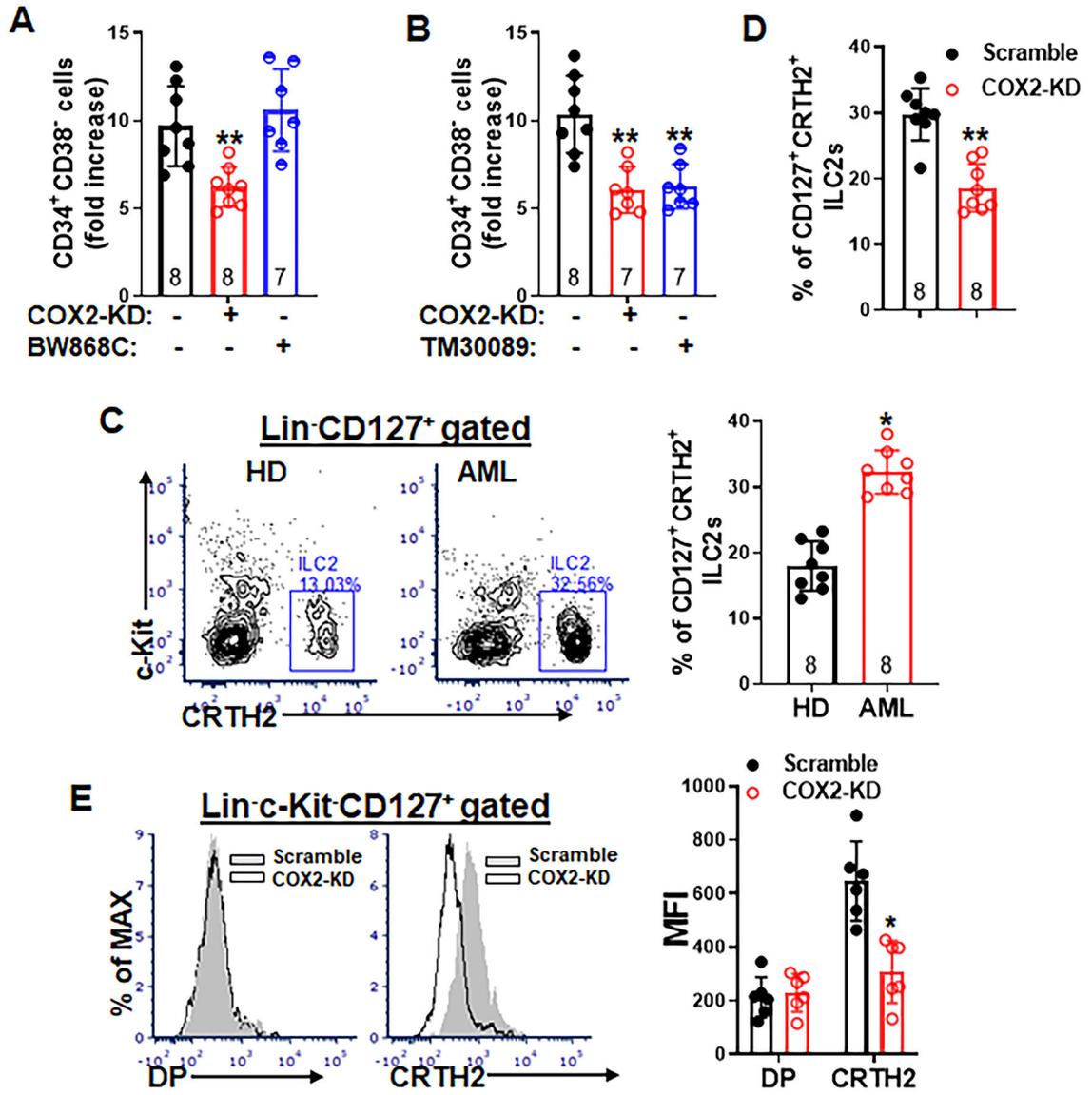
Fig 2. CRTH2/DP2 mediates the effect of mesenchymal PGD$_2$ in HSPC expansion.
(A) DP antagonist fails to block HSPC expansion. 1,000 normal BM CD34$^+$ cells were co-cultured with AML MSCs transduced with scramble shRNA or shRNA targeting *COX2* in the presence or absence of DP antagonist, BW868C (1 μM). CD34$^+$CD38$^-$ cells derived from the co-cultured CD34$^+$ HSPCs were analyzed by flow cytometry at day 4. Results are means ± SD of three independent experiments (n=7–8 for each group). (B) CRTH2 antagonist mimics *COX2* knockdown and inhibits HSPC expansion. 1,000 normal BM CD34$^+$ cells were co-cultured with AML MSCs transduced with scramble shRNA or shRNA targeting *COX2* in the presence or absence of TM30089 (1 μM). CD34$^+$CD38$^-$ cells derived from the co-cultured CD34$^+$ HSPCs were analyzed by flow cytometry at day 4. Results are means ± SD of three independent experiments (n=7–8 for each group). (C) AML MSCs expands CD127$^+$CRTH2$^+$ILC2s in the progeny of co-cultured CD34$^+$ cells. 1,000 normal BM CD34$^+$ cells were co-cultured with MSCs from HD or AML patients followed by flow

cytometry analysis for the frequency of CD127$^+$CRTH2$^+$ILC2s at day 4. Representative flow plots (Left) and quantification (Right) are shown. (D) *COX2*-KD reduces the expansion of CD127$^+$CRTH2$^+$ILC2s. 1,000 normal BM CD34$^+$ cells were co-cultured with AML-MSCs expressing scramble shRNA or shRNA targeting *COX2* followed by flow cytometry analysis for the frequency of CD127$^+$CRTH2$^+$ILC2 at day 4. Results are means ± SD of three independent experiments (n=8 for each group). (E) *COX2*-KD reduces CRTH2 expression. Cells described in (D) were subjected to flow cytometry analysis for DP and CRTH2. Lin$^-$c-kit$^+$CD127$^+$ cells were gated for analysis. Representative histogram (Left) and quantification of mean fluorescence intensity (MFI; Right) are shown. Results are means ± SD of three independent experiments (n=8 for each group).
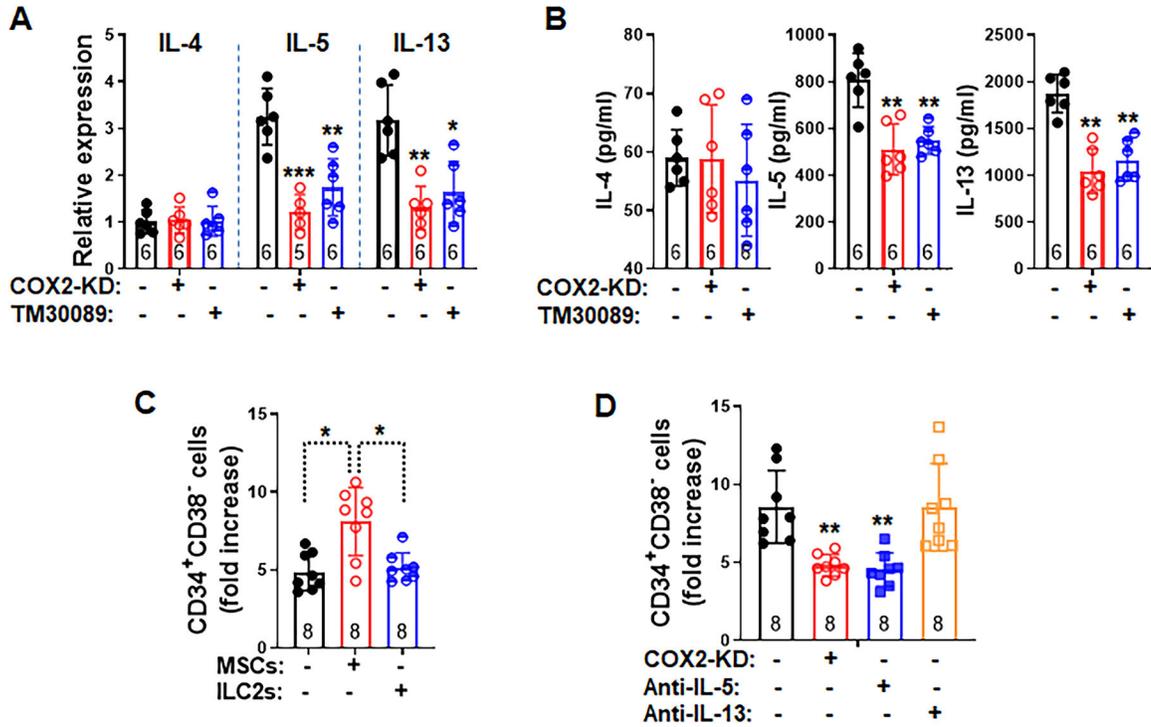
Fig 3. Activation of mesenchymal PGD$_2$-CRTH2/ILC2 axis overproduces IL-5, which promotes HSPC expansion.

(A, B) TM30089 treatment reduces the levels of IL-5 and IL-13 in co-cultured CD127$^+$CRTH2$^+$ILC2s. 10,000 CD127$^+$CRTH2$^+$ILC2s were co-cultured with AML MSCs expressing scramble or *COX2* shRNA with or without TM30089 for 48 h followed by qPCR (A; normalized to the level of *GAPDH* mRNA) using primers listed in Table S2, or immunosorbent analysis (B) for IL-4, IL-5 and IL-13. Results are means ± SD of three independent experiments (n=5–6 for each group). (C) CD127$^+$CRTH2$^+$ ILC2s alone fail to promote HSPC expansion. 1,000 normal BM CD34$^+$ cells were cultured in the presence or absence of AML-MSCs, or sorted CD127$^+$CRTH2$^+$ILC2s followed by flow cytometry analysis for CD34$^+$CD38$^-$ cells derived from the co-cultured CD34$^+$ HSPCs on day 4. Results are means ± SD of three independent experiments (n=8 for each group). (D) IL-5 promotes co-cultured BM HSPC expansion. 1,000 normal BMCD34$^+$ cells were co-cultured with AML MSCs expressing scramble shRNA or shRNA targeting *COX2*, with or without antibodies against IL5 or IL13 followed by flow cytometry analysis for CD34$^+$CD38$^-$ cells derived from the co-cultured CD34$^+$ HSPCs on day 4. Results are means ± SD of three independent experiments (n=8 for each group).
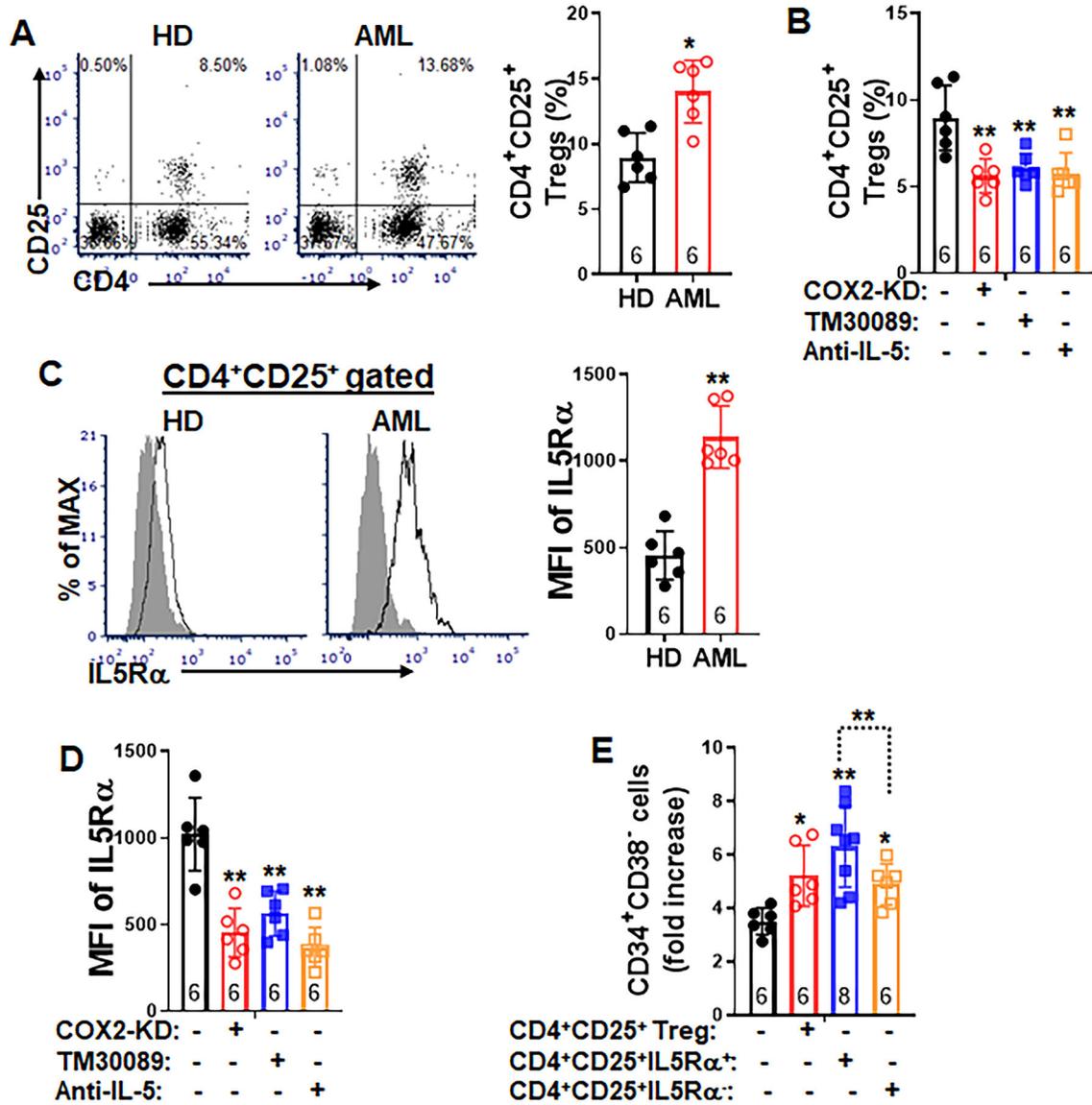
Fig 4. Mesenchymal PGD2-activated ILC2s-induced IL-5 expands CD4$^+$CD25$^+$IL5Rα^+ Tregs, which promote HSPC expansion.

(A) AML-MSCs expands CD4$^+$CD25$^+$ Tregs. 1,000 normal BM CD34$^+$ cells were co-cultured with AML MSCs or HD MSCs followed by flow cytometry analysis for CD4$^+$CD25$^+$ Tregs derived from the co-cultured CD34$^+$ HSPCs on day 4. Representative flow plots (Left) and quantification (Right) are shown. Results are means ± SD of three independent experiments (n=6 for each group). (B) AML MSCs-induced expansion of CD4$^+$CD25$^+$ Tregs is abolished by inhibition of the mesenchymal PGD2-CRTH2/ILC2s axis. 1,000 normal BM CD34$^+$ cells were co-cultured on AML MSCs expressing scramble or *COX2* shRNA, with or without TM30089 or anti-IL-5 antibody followed by flow cytometry analysis for CD4$^+$CD25$^+$ Tregs derived from the co-cultured CD34$^+$ HSPCs on day 4. Results are means ± SD of three independent experiments (n=6 for each group). (C) AML MSCs-expanded Tregs express high levels of IL-5Rα. CD4$^+$CD25$^+$ cells described in (A) were gated for IL-5Rα analysis by flow cytometry. Representative histogram (Left) and

quantification (right) are shown. Results are means ± SD of three independent experiments (n=6 for each group). (D) AML MSCs-induced expression of IL-5Rα in Tregs is abolished by inhibition of the mesenchymal PGD2-CRTH2/ILC2s axis. CD4$^+$CD25$^+$ cells described in (B) were gated for IL-5Rα expression. Results are means ± SD of three independent experiments (n=6 for each group). (E) CD4$^+$CD25$^+$IL5Rα$^+$ Tregs exhibit higher potential in promoting co-cultured HSPC expansion. CD4$^+$CD25$^+$ IL-5Rα$^+$ or CD4$^+$CD25$^+$ IL-5Rα$^-$ Tregs were sorted for co-culture with normal CD34$^+$ cells followed by flow cytometry analysis for CD34$^+$CD38$^-$ cells derived from the co-cultured CD34$^+$ HSPCs on day 4. Results are means ± SD of three independent experiments (n=6–8 for each group).
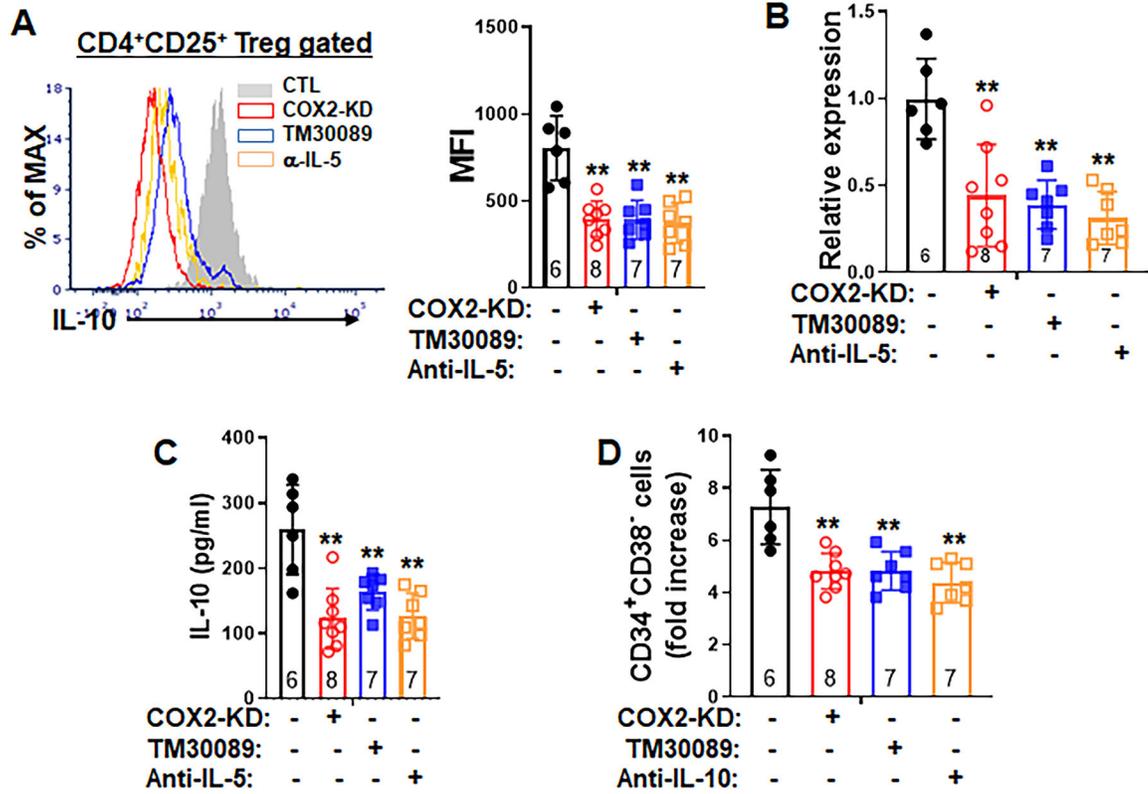
Fig 5. Mesenchymal PGD$_2$-expanded CD4$^+$CD25$^+$ Tregs produce IL-10, which promotes co-cultured HSPC expansion.

(A) Intra-cellular levels of IL-10 in AML MSC/PGD$_2$-expanded Tregs. 1,000 normal BM CD34$^+$ cells were co-cultured on AML MSCs expressing scramble shRNA or shRNA targeting *COX2*, with or without TM30089 or anti-IL-5 antibody followed by flow cytometry analysis for intra-cellular IL-10 in CD4$^+$CD25$^+$ Tregs derived from the co-cultured CD34$^+$ HSPCs on day 4. Representative histogram (Left) and quantification (Right) are shown. Results are means ± SD of three independent experiments (n=6–8 for each group). (B, C) AML MSC/PGD$_2$-driven expression of IL-10 in Tregs is abolished by inhibition of the mesenchymal PGD2-CRTH2/ILC2s axis. 1,000 normal BM CD34$^+$ cells co-cultured on AML-MSCs expressing scramble shRNA or shRNA targeting *COX2*, with or without TM30089 or anti-IL-5 for 4 days. CD4$^+$CD25$^+$ Tregs were then sorted for qPCR (B) or ELISA (C) analysis for IL-10. (D) AML MSC/PGD$_2$-induced IL-10 promotes expansion of co-cultured CD34$^+$CD38$^-$ HSPCs. 1,000 normal BM CD34$^+$ cells were co-cultured with AML-MSCs expressing scramble shRNA or shRNA targeting *COX2*, with or without TM30089 or anti-IL-10 antibody followed by flow cytometry analysis for CD34$^+$CD38$^-$ cells derived from the co-cultured CD34$^+$ HSPCs on day 4. Results are means ± SD of three independent experiments (n=6–8 for each group).
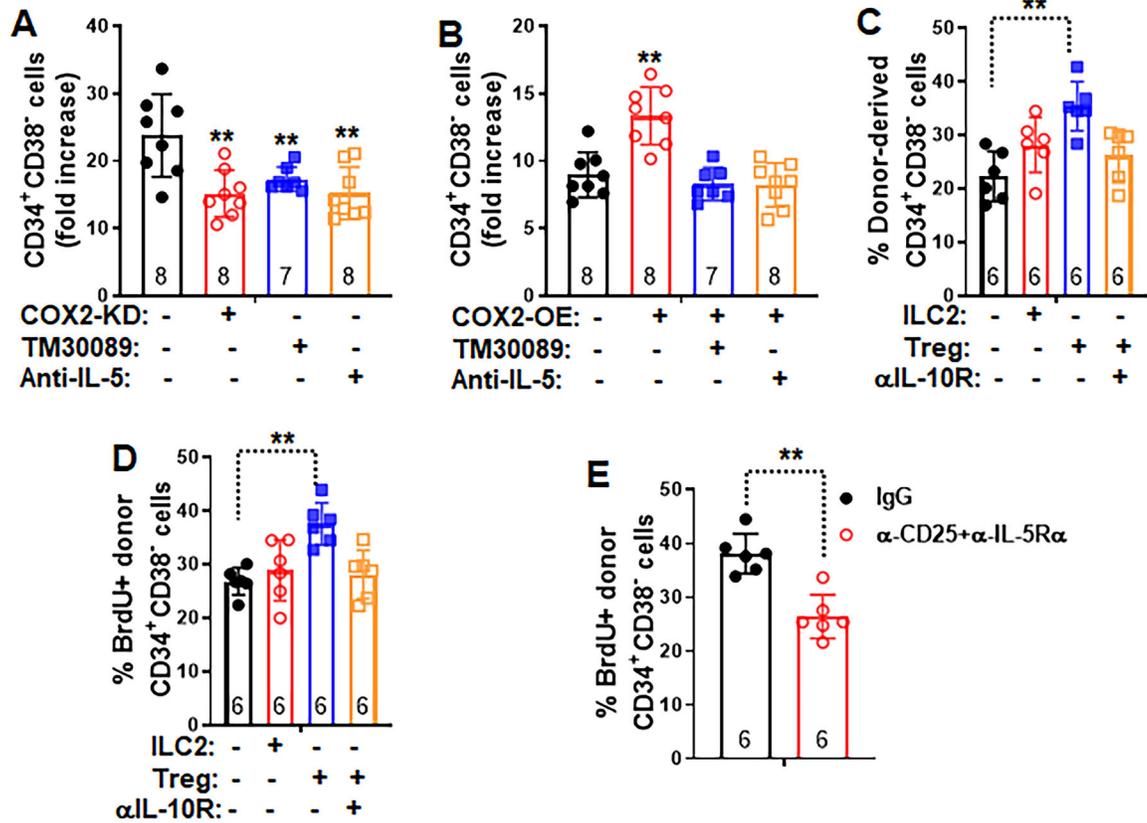
Fig 6. Mesenchymal PGD2-activated ILC2-Treg axis promotes proliferation of normal HSPCs.
(A) AML MSC/PGD$_2$-driven expansion of AML CD34$^+$CD38$^-$ cells is abolished by inhibition of the mesenchymal PGD2-CRTH2/ILC2s axis. 1,000 BM CD34$^+$ cells from AML patients were co-cultured on AML MSCs expressing scramble shRNA or shRNA targeting *COX2*, with or without TM30089 or anti-IL-5 antibody followed by flow cytometry analysis for CD34$^+$CD38$^-$ cells derived from the co-cultured CD34$^+$ HSPCs on day 4. Results are means ± SD of three independent experiments (n=7–8 for each group). (B) Ectopic expression of COX2 in HD MSCs promotes expansion of AML CD34$^+$CD38$^-$ cells. 1,000 BM CD34$^+$ cells from AML patients were co-cultured on HD MSCs expressing EV or *COX2*, with or without TM30089 or anti-IL-5 antibody followed by flow cytometry analysis for CD34$^+$CD38$^-$ cells derived from the co-cultured CD34$^+$ HSPCs on day 4. Results are means ± SD of three independent experiments (n=7–8 for each group). (C) Mesenchymal PGD2-activated ILC2-Treg axis promotes expansion of normal HSPCs *in vivo*. 2,000 normal BM CD34$^+$ cells were co-transplanted with 30,000 Lin$^-$CD127$^+$CRTH2$^+$ ILC2s or CD4$^+$CD25$^+$IL-5Rα^+ Tregs isolated from the co-cultures directly into the right femurs of sublethally irradiated NSGS recipient mice, which were injected intraperitoneally (i.p.) with anti-IL-10R or control IgG antibody (200 μg/mouse) every other day from day −1 until day 7 post-transplant. Donor-derived CD34$^+$CD38$^-$ cells were determined by flow cytometry 6 weeks post-transplant by BM aspiration (n=6 for each group). (D) Mesenchymal PGD2-activated ILC2-Treg axis promotes proliferation of normal HSPCs *in vivo*. The recipients described in (C) were i.p. injected with BrdU (150 μl of 10 mg/ml). Whole bone marrow cells (WBMCs) were aspirated for BrdU incorporation assay 14 hours

later. hCD34$^+$CD38$^-$ cells were gated for analysis for BrdU-positive cells (n=6 for each group). (E) CD4$^+$CD25$^+$IL5Rα$^+$ Tregs promote proliferation of normal HSPCs in secondary NSGS recipient mice. 2,000 normal CD34$^+$ cells were co-transplanted with 30,000 CD4$^+$CD25$^+$IL-5Rα$^+$ Tregs isolated from the co-cultures directly into the right femurs of sublethally irradiated NSGS recipient mice. Six weeks later, the primary recipient mice were sacrificed; 3×10^6 BM cells were transplanted into secondary NSGS recipient mice. These secondary recipient mice were then treated with control IgG or anti-CD25 and anti-IL-5Rα antibodies (250 μg/mouse, single dose, i.p.) to deplete CD4$^+$CD25$^+$IL-5Rα$^+$ Tregs. The recipients were i.p. injected with BrdU (150 μl of 10 mg/ml). WBMCs were aspirated for BrdU incorporation assay 14 hours later. hCD34$^+$CD38$^-$ cells were gated for analysis for BrdU-positive cells (n=6 for each group).
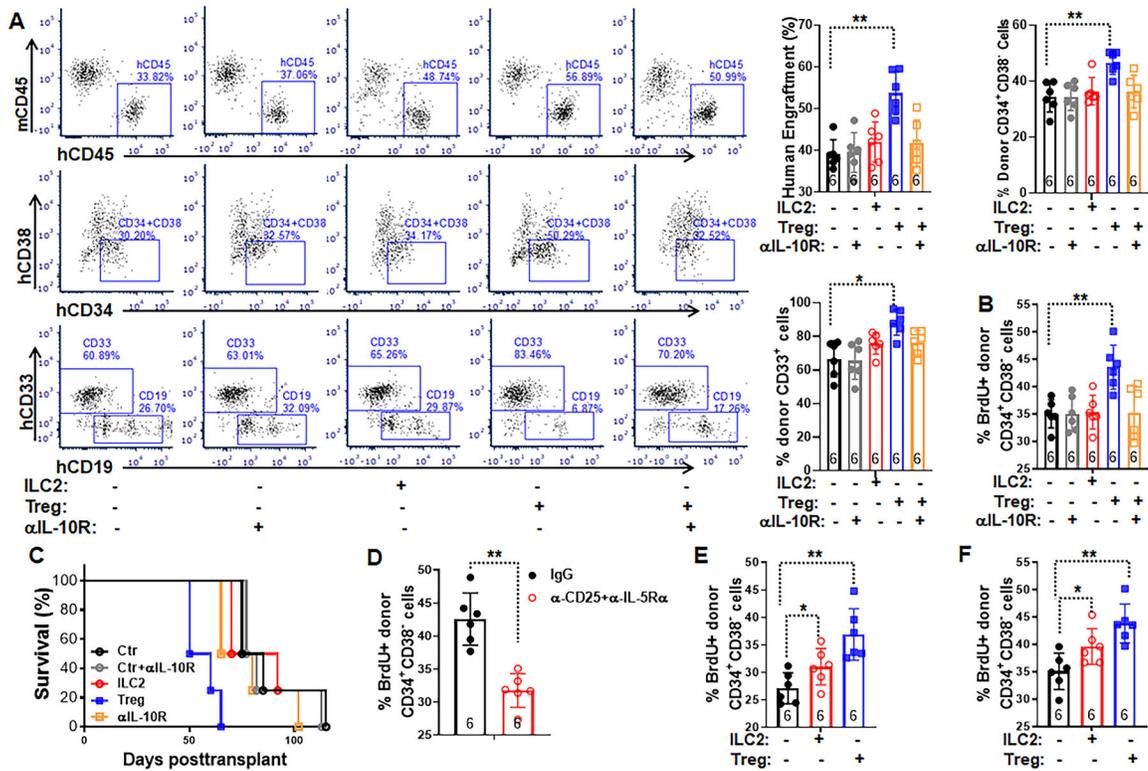
Fig 7. Mesenchymal PGD2-activated ILC2-Treg axis promotes proliferation of malignant HSPCs.

(A) Mesenchymal PGD2-activated ILC2-Treg axis promotes expansion of malignant HSPCs *in vivo*. 2,000 CD34$^+$ cells from AML patients were co-transplanted with 30,000 Lin$^-$CD127$^+$CRTH2$^+$ ILC2s or CD4$^+$CD25$^+$IL-5Rα^+ Tregs isolated from the co-cultures directly into the right femurs of sublethally irradiated NSGS recipient mice, which were injected i.p. with anti-IL-10R or control IgG antibody (200 μg/mouse) every other day from day −1 until day 7 post-transplant. Whole bone marrow cells (WBMCs) were aspirated for flow cytometry analysis for total human engraftment (hCD45; upper); CD34$^+$CD38$^-$ (middle) and myeloid (CD33$^+$)/lymphoid (CD19$^+$) (lower) cells were determined 6 weeks post-transplant. Representative flow plots of (Left) and quantification (Right) are shown (n=6). (B) Mesenchymal PGD2-activated ILC2-Treg axis promotes proliferation of AML HSPCs *in vivo*. The recipients described in (A) were i.p. injected with BrdU. Whole bone marrow cells (WBMCs) were aspirated for BrdU incorporation assay 14 hours later. hCD34$^+$CD38$^-$ cells were gated for analysis for BrdU-positive cells (n=6 for each group). (C) Mesenchymal PGD2-activated ILC2-Treg axis accelerates leukemia development in NSGS recipient mice. Survival of the recipients described in (A) was plotted by the Kaplan-Meier method and analyzed by the log-rank test. (D) CD4$^+$CD25$^+$IL5Rα^+ Tregs promote proliferation of malignant HSPCs in secondary NSGS recipient mice. 2,000 AML CD34$^+$ cells were co-transplanted with 30,000 CD4$^+$CD25$^+$IL-5Rα^+ Tregs isolated from the co-cultures directly into the right femurs of sublethally irradiated NSGS recipient mice. Six weeks later, the primary recipient mice were sacrificed; 3×10^6 BM cells were transplanted into secondary NSGS recipient mice. These secondary recipient mice were then treated with control IgG or anti-CD25 and anti-IL-5Rα antibodies (250 μg/mouse, single dose, i.p.) to

deplete CD4$^+$CD25$^+$IL-5Rα^+ Tregs. The recipients were i.p. injected with BrdU (150 μl of 10 mg/ml). WBMCs were aspirated for BrdU incorporation assay 14 hours later. hCD34$^+$CD38$^-$ cells were gated for analysis for BrdU-positive cells (n=6 for each group). (E, F) Leukemic Lin$^-$CD127$^+$CRTH2$^+$ ILC2s and CD4$^+$CD25$^+$IL-5Rα^+ Tregs promote the expansion of normal and malignant HSPCs in xenotransplanted recipient mice. 2,000 normal BM CD34$^+$ cells (E) or 2,000 AML CD34$^+$ cells (F) were co-transplanted with or without 30,000 leukemic Lin$^-$CD127$^+$CRTH2$^+$ ILC2s or CD4$^+$CD25$^+$IL-5Rα^+ Tregs isolated from the leukemic mice described in Figure 6E, into the right femurs of sublethally irradiated NSGS recipient mice. Two weeks post-transplant, the recipients were i.p. injected with BrdU (150 μl of 10 mg/ml). WBMCs were aspirated for BrdU incorporation assay 14 hours later. hCD34$^+$CD38$^-$ cells were gated for analysis for BrdU-positive cells (n=6 for each group).
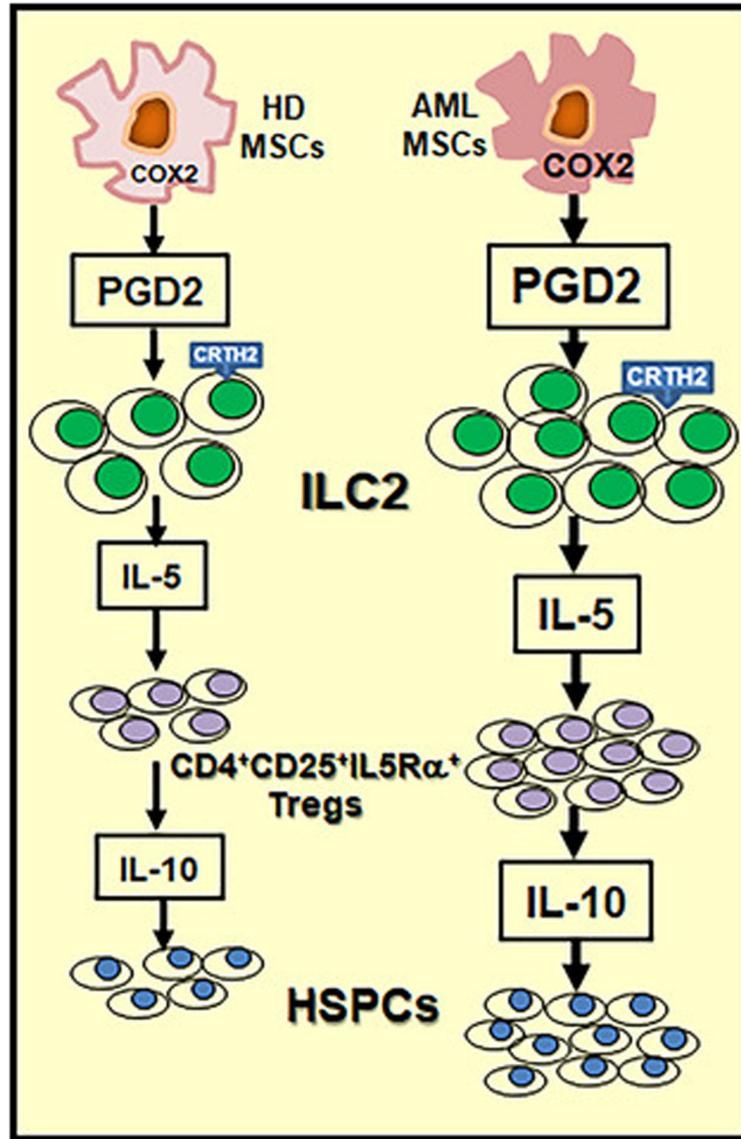
Fig 8. Mesenchymal PGD2 activates an ILC2-Treg axis to promote proliferation of normal and malignant HSPCs.

Mesenchymal COX2-derived PGD2 engages the receptor CRTH2 on ILCs to promote the expansion of ILC2s. The ILC2s overproduce IL-5, which specifically expands $CD4^+CD25^+IL5R\alpha^+$ Tregs. The expanded $CD4^+CD25^+$ $IL5R\alpha^+$ Tregs produce IL-10, which promotes HSPC proliferation.