ARTICLE

PPARγ drives IL-33-dependent ILC2 pro-tumoral functions

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Group 2 innate lymphoid cells (ILC2s) play a critical role in protection against helminths and in diverse inflammatory diseases by responding to soluble factors such as the alarmin IL-33, that is often overexpressed in cancer. Nonetheless, regulatory factors that dictate ILC2 functions remain poorly studied. Here, we show that peroxisome proliferator-activated receptor gamma (PPARγ) is selectively expressed in ILC2s in humans and in mice, acting as a central functional regulator. Pharmacologic inhibition or genetic deletion of PPARγ in ILC2s significantly impair IL-33-induced Type-2 cytokine production and mitochondrial fitness. Further, PPARγ blockade in ILC2s disrupts their pro-tumoral effect induced by IL-33-secreting cancer cells. Lastly, genetic ablation of PPARγ in ILC2s significantly suppresses tumor growth in vivo. Our findings highlight a crucial role for PPARγ in supporting the IL-33 dependent pro-tumorigenic role of ILC2s and suggest that PPARγ can be considered as a druggable pathway in ILC2s to inhibit their effector functions. Hence, PPARγ targeting might be exploited in cancer immunotherapy and in other ILC2-driven mediated disorders, such as asthma and allergy.

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innate lymphoid cells (ILCs) are the most recently identified subset of innate lymphocytes. ILCs can be classified in three principal groups according to their transcription factor expression and cytokine secretion, mirroring to some extent CD4 T helper subsets1. Group 1 ILCs (ILC1s) express T-bet and secrete IFN-γ. Group 2 ILCs (ILC2s) express GATA3 and secrete IL-13 and IL-5. Tissue-resident group 3 ILCs (ILC3s) express RORγT and secrete IL-22 and IL-17 while, in the human peripheral circulation, ILC3s also comprise a population of progenitor cells (ILCPs) able to differentiate into all ILC subsets and natural killer (NK) cells2. In the last few years, different reports highlighted the important contribution of ILCs to different physiological and pathophysiologic processes3. In particular, ILC2s have been reported to play a critical role in several inflammatory diseases including asthma, chronic rhinosinusitis, and allergic rhinitis4–6. Moreover, we and others have shown that ILC2s mediate tumor immune responses, with either pro- or anti-tumor effects depending on the tumor type7–11. Nonetheless, external and cell-intrinsic regulatory factors that dictate ILC2 activation and function remain poorly studied, particularly in humans. Better knowledge on ILC2-drivers is warranted to enable pharmacological interference with ILC2 functions in disease settings. In that regard, alarmins (e.g., IL-33) were shown to directly activate ST2-expressing ILC2s and induce their Type-2 cytokine secretion12. Further, it was reported that certain types of lipids or lipid-derivatives act as key mediators involved in ILC2 activation. In particular, in the context of helminth infections, ILC2 function is dependent on fatty acid synthesis and oxidation, which is known to be regulated by peroxisome proliferator-activated receptors (PPARs)13,14. PPARs are a class of nuclear hormone receptors that act as transcription factors regulating gene expression15. They are divided in three major isoforms: α, β/δ, and γ that differ in their tissue expression and functions15. PPARγ regulates the transcription of genes associated with lipid metabolism and is expressed in different immune cells, including lymphocytes, monocytes, dendritic cells, and platelets, where it mainly exerts anti-inflammatory effects16. Although different synthetic PPARγ ligands are used in the clinics, 15-deoxy-A12-14-PGJ2 (15d-PGJ2) is one of the few mediators that is often referred as an endogenous PPARγ ligand17. 15d-PGJ2 is a prostaglandin D2 (PGD2)-derived product that has been reported to be essential for ILC2 activation18–20. In particular, it has recently been shown that PGD2 and its metabolites (including 15d-PGJ2) are endogenously synthesized after ILC2 stimulation with IL-33, IL-25, and TSLP19. Furthermore, elevated levels of PPARγ and other genes involved in prostaglandin (PG) synthesis and response were recently reported in a single cell mRNA-sequencing analysis of ILC2s sorted from human tonsil tissue21. In this work, we characterize the expression and functional role of PPARγ in human and mouse ILCs to assess whether PPARγ can be targeted in the context of an ILC-directed immunotherapy.

Results

Ex vivo and in vitro expanded human ILC2s specifically express PPARγ. In order to characterize ILC2s in terms of function and activation, we mined a previously performed RNA-sequencing (RNA-seq) analysis of ILC1s, ILC2s, and ILCPs sorted from healthy donor (HD) PBMCs, and compared their transcriptional signature (ArrayExpress E-MTAB-8494). In particular, focusing on fatty acid metabolism-related genes, that have been associated to ILC2 response during helminthic infection, we found that PPARγ (PPARG) was more expressed in ILC2s, as compared to the other ILC subsets (Fig. 1a, left panel), while no differences in PPARα (PPARA) and PPARβ (PPARD) expression were observed among the subsets (Fig. 1a, right panel), as also confirmed by qPCR (Fig. 1b). Similar observations were made by the analysis of PPAR expression in in vitro expanded ILC subsets, both at mRNA and protein level (Fig. 1c, d). Given that ILCs are considered as the innate mirrors of CD4 T helper subsets, we analyzed the expression of PPARs in the adaptive counterparts of ILC1s, ILC2s, and ILCPs, i.e., Th1, Th2, and Th17 cells, respectively. PPARγ expression was preferentially observed in human Th2 and Th17 but not Th1 cells upon expansion (Supplementary Fig. 1a, d), in contrast to the mouse where PPARγ protein is expressed predominantly in Th2 cells22. Taken together, these findings show specific expression of PPARγ in human ILC2s, which is also maintained during in vitro culture.

PPARγ sustains Type-2 cytokine secretion in human ILC2s. To better elucidate the functional role of PPARγ in ILC2s, we analyzed the cytokine production of IL-33/IL-25 (referred as IL) stimulated ILC2s, after pre-incubation with non-toxic concentrations of T0070907, a selective and irreversible PPARγ antagonist23. As shown in Fig. 2a, b, ILC2s treated with T0070907 produced significantly less IL-13 as compared to control ILC2s. Moreover, a trend for reduction was also observed for other ILC2-specific cytokines, including IL-4 and amphiregulin (AREG) (Fig. 2b)24. These results were confirmed by the quantification of cytokines in cell culture supernatants of ILC2s, pretreated or not with T0070907 (Fig. 2c). This additional readout allowed us to observe that T0070907 not only reduced the secretion of IL-13 in a concentration-dependent manner (Supplementary Fig. 2a), but also of IL-5 in pre-treated ILC2s (Fig. 2c). To test if the inhibitory effects of T0070907 were due to the selective inhibition of PPARγ, we transdifferentiated human in vitro expanded ILC2s with a small interfering RNA (siRNA) targeting PPARγ. The knockdown of PPARγ was confirmed by qPCR analysis (Supplementary Fig. 2b). Consistent with the impact of pharmacological inhibition, PPARγ silencing led to significantly reduced cytokine production and secretion (Supplementary Fig. 2c–e). This effect on Type-2 cytokine secretion might be at least in part a direct consequence of PPARγ binding to peroxisome proliferator response elements (PPREs), since we found motives partially matching the human PPARγ-retinoid X receptor alpha (RXRa) heterodimer binding motif in the promoter region of both IL-5 and IL-13 genes (Fig. 2d, Supplementary Data 1). To substantiate this finding, we performed Chromatin immunoprecipitation (ChiP) on human expanded ILC2s stimulated with the cytokine cocktail and treated or not with T0070907. The results indicated that PPARγ directly bound to the promoter of IL-13 in cytokine-activated ILC2s (Supplementary Fig. 2f). An increasing number of reports demonstrated a direct correlation between PG synthesis and PPARγ activation25–27. In particular, it has been recently reported that the inhibition of the cyclooxygenase (COX), which is the key rate-limiting enzyme in PG production, prevents IL-5 and IL-13 secretion by cytokine-stimulated ILC2s19. Based on these data, we speculated that COX and PPARγ could exert a synergistic effect on ILC2 cytokine secretion. Hence, we treated ILC2s with a combination of T0070907 and celecoxib (CBX), a selective inhibitor of COX-2, a gene upregulated in ILC2s after cytokine stimulation18. Although not statistically significant, we observed that this treatment induced a partial reduction in IL-13 and IL-5 secretion (Supplementary Fig. 2g).
Fig. 1 Human ex vivo and in vitro expanded ILC2s specifically express PPARγ. a Heatmap of row z-scores of mRNA level of proteins involved in fatty acid metabolism (highlighted in italics) or involved in protein-protein interaction with PPARγ (highlighted in bold). Fatty acid metabolism genes were taken from the “fatty acid metabolic process” Gene Ontology gene set (GO:0006631, http://geneontology.org/). RNA-sequencing counts were transformed to log2 counts per million, and differential gene expression analysis was performed by fitting a linear model to each gene using the voom function in the limma package for R (v. 3.38.3), followed by a moderated t statistic computation using the empirical Bayes statistics model implemented in the limma package (See Ercolano et al.1 for detailed methods). Genes significantly upregulated in ILC2s compared to ILC1s and/or ILCPs are indicated using an * (*n = 3; *P < 0.05). Proteins putatively involved in protein–protein interactions were taken from the STRING database (https://string-db.org/). b Expression of PPARs assessed by qPCR in human freshly sorted ILC subsets (open square ILC1s, open circle ILC2s, open triangle ILCPs) (ILC1s n = 6; ILC2s and ILCPs n = 7; ILC1s vs ILC2s **P = 0.0032, ILC2s vs ILCPs **P = 0.0021). c Expression of PPARs assessed by qPCR in in vitro expanded human ILC subsets (open square ILC1s, open circle ILC2s, open triangle ILCPs) (ILC1s and ILCPs n = 3; ILC2s n = 5; ILC1s vs ILC2s ****P < 0.0001, ILC2s vs ILCPs *P = 0.0020). d Western blot analysis of PPARγ expression at protein level in ILC2s compared to ILC1s and ILCPs (one individual experiment). Each symbol represents one individual donor. Data are shown as mean ± SEM and were analyzed by one- (b) or two-way (c) ANOVA tests. Source data are provided as a Source data file.
inhibition of ILC2s, we measured mitochondrial mass by MitoTracker Green uptake and MitoTracker Deep Red fluorescence in these cells. As shown in Fig. 3a–d, both MitoTracker Green and Deep Red dye uptake were decreased in ILC2s after treatment with the PPARγ inhibitor, suggesting reduced mitochondrial mass. We also used tetramethylrhodamine methyl ester (TMRM) that, by accumulating in intact and active mitochondria, allows to assess changes in their membrane potential. Consistent with the MitoTracker results, the TMRM assay showed a significant decrease of mitochondrial potential after incubation of ILC2s with T0070907 (Fig. 3e, f). To corroborate our data, we performed electron microscopy (EM) analysis that showed reduced percentage of mitochondrial volume per cell in T0070907-treated ILC2s as compared to control (Fig. 3g, h). These findings indicate that the inhibitory effects of T0070907 correlates with a decline in mitochondrial function, which in turn likely impairs ILC2 cellular fitness.

**Tumor-derived IL-33 sensitizes ILC2s to produce IL-13.** Over the last decade, alarmins have been identified as signaling mediators involved in cancer development and progression. In particular, different studies reported that IL-33 and its receptor ST2 play a critical role in the pathogenesis of different types of cancers, including breast cancer, hepatocellular carcinoma, and colorectal cancer (CRC). We used CRC as a model to address a putative role of tumor-derived IL-33 on ILC2 functions in the context of tumor immunity. First, we confirmed expression of IL-33 in human CRC tissues using a Tissue Micro Array (Fig. 4a). GATA3+CD3− cells were identified in proximity to IL-33+ CRC epithelial cells, suggesting that IL-33 released from these malignant cells may stimulate ILC2s in human CRC lesions (Fig. 4b). In line with these findings, we observed elevated IL-33 levels in the serum of CRC patients as compared to HDs (Fig. 4c). Next, we evaluated ILC2 frequency and function, in terms of cytokine production, in both PBMCs and tumor-infiltrating lymphocytes (TILs) in CRC patients. As previously reported, circulating ILC2 frequency was comparable in HDs and CRC patients (Fig. 4d). Importantly, ILC2s are present among TILs, as assessed by flow cytometry analysis of dissociated CRC tissues (Fig. 4d). In terms of function, ILC2s from CRC patients’ PBMCs produced more IL-13 compared to ILC2s from HDs’ PBMCs (Fig. 4e, f). To gain further insight into the role of PPARγ in ILC2s in the CRC setting, we evaluated the expression of PPARγ, as well as of CPT1A, one of the well-characterized PPARγ-direct target genes, in freshly-sorted ILC2s from both PBMCs and TILs of CRC patients. As shown in Supplementary Fig. 3a, b, a trend for higher expression of PPARγ and CPT1A was observed in CRC patients’ PBMCs compared to HDs, suggesting that PPARγ is not only increased but also more active in the CRC setting. In addition, exposure of expanded ILC2s from CRC patients to T0070907 resulted in the reduction of IL-13 and IL-5 secretion as well as in impairment of mitochondrial functions, thus recapitulating the effects observed in HD ILC2s (Supplementary Fig. 3c–e). Collectively, these results indicate that ILC2s from cancer patients are more prone to secrete cytokines, which may be related to their increased exposure to IL-33, both systemically and in the tumor microenvironment.

**IL-13 drives the crosstalk between ILC2s and cancer cells.** In the last few years, IL-13 and its receptors have been identified as novel targets for cancer therapy, and inhibition of IL-13-producing cells as a strategy to reach this goal. To better
understand the crosstalk between PPARγ, ILC2s, and the CRC cells, we pre-treated ILC2s with T0070907 or solvent-control and co-cultured them with the SW1116 CRC cell line. As shown in Fig. 5a, b, the ILC2-SW1116 co-culture resulted in increased IL-13 production by ILC2s that was abolished by pre-treating ILC2s with T0070907. IL-13 production in ILC2s was possibly triggered by increased expression of IL-33 in co-cultured CRC cells (Fig. 5c). To address this hypothesis, we performed coculture experiments using the helminth parasite-secreted protein (HpARI), known to suppress type 2 immune responses through the interference with the IL-33 pathway. As shown in Supplementary Fig. 4a, the addition of HpARI in the medium significantly reduced the production of IL-13 by ILC2s, after co-culture with SW1116 colorectal cancer cells. Since IL-13 is involved in CRC progression and metastasis development by affecting epithelial to mesenchymal transition (EMT), we hypothesized that ILC2-derived IL-13 could affect EMT in CRC cells. To address this point, we performed wound healing and clonogenic assays using conditioned medium (CM) of activated ILC2s, pre-treated or not with T0070907 (ILC2 CM and ILC2 + T0070907, respectively). No effect was observed on the CRC cell proliferation (Supplementary Fig. 4b). However, we found that the presence of ILC2 CM increased the migration of SW1116 cells, whereas addition of ILC2 + T0070907 CM lead to the opposite effect (Fig. 5d, e). Likewise, the colony formation assay confirmed that ILC2 CM increased the number of SW1116 colonies compared to control and to ILC2 + T0070907 CM (Fig. 5f). Moreover, the addition of an anti-IL-13 blocking antibody abrogated the development of SW1116 colonies, confirming the involvement of IL-13 in the ILC2-PPARγ-dependent effect on CRC cells (Fig. 5f). In order to corroborate our findings on the pro-tumor effects of ILC2 CM for the migration and invasion of CRC, we evaluated in the SW116 line the expression of MMP9 and N-cadherin, two molecules that have been linked to the progression and invasion of tumor cells. We found that the addition of ILC2 CM significantly increased the expression of these two EMT markers on CRC cells, whilst the addition of ILC2 + T0070907 CM or the anti-IL-13 blocking antibody reduced their expression towards basal levels (Fig. 5g). Taken together, these data suggest a crosstalk between cancer cells and ILC2s, in which cancer cells trigger ILC2s to produce IL-13, which in turn sustains their migratory and invasive capacity, yet without affecting their proliferation rate. Inhibition of PPARγ in this circuit thwarts the pro-tumorigenic role of ILC2s.

PPARγ is expressed and functional in murine ILC2s. To translate our findings into an in vivo setting, we assessed the expression of PPARγ in freshly-sorted ILC subsets from the lung of C57BL/6 mice. In agreement with our human data, PPARγ was specifically expressed in ILC2s, while it was not detectable in ILC1s and ILC3s (Fig. 6a). Therefore, we exposed murine ILC2s to T0070907 in vitro and could thereby recapitulate the results obtained using human ILC2s, with a reduction in the production of both IL-13 and IL-5 (Fig. 6b, c). Next, to define the role of PPARγ in ILC2s beyond its pharmacological inhibition, we used a transgenic mouse model with an ILC-specific PPARγ inducible knockout (KO) by crossing Ppargfl/fl Id2CreERT2 positive or negative mice (also reported as Ppargfl/fl Id2Cre + and Ppargfl/fl Id2Cre, respectively) with Il33 and Il-25 i.p. for 3 days, after 5 days of tamoxifen injection. As shown in Fig. 6e, ILC2s...
sorted from Ppargfl/fl Id2Cre+ mice secreted significantly less IL-13 and IL-5 compared to control Ppargfl/fl Id2Cre− mice. In addition, similarly to the results observed in humans, we found motives partially matching the mouse PPARγ-Rxra heterodimer binding motif in the promoter region of both Il-5 and Il-13 genes (Fig. 6f, Supplementary Data 2). Taken together these results confirm the role of PPARγ as a key regulator of ILC2 function in vivo.

**PPARγ expression in ILC2s influences cancer progression in a CRC murine model.** Next, to investigate the in vivo role of ILC2s in CRC development and progression, we used a heterotopic murine model of CRC in RORegfl/flIl7rCre mice (referred to as ILC2 KO mice)13. We injected subcutaneously (s.c.) MC-38 murine genetically modified CRC cells to constitutively produce IL-33 (reported as MC-38-IL33). Tumor cells were injected into the right flank of ILC2 KO and littermate control mice, and survival was monitored. As displayed in Fig. 7a, tumor progression was slower in ILC2 KO mice compared to littermate controls, resulting in better survival advantage in the KO strain, suggesting a pro-tumorigenic function of ILC2s in this model. This effect was reverted after the adoptive transfer of expanded ILC2s sorted from the lungs of C57BL6 mice (Fig. 7b). Next, to further assess the contribution of PPARγ to ILC2s during tumorigenesis, we injected MC-38-IL33 cells into Ppargfl/fl Id2Cre+ mice and Ppargfl/fl Id2Cre− mice (Fig. 7c). Strikingly, after 18 days, tumor volume was reduced in Ppargfl/fl Id2Cre+ mice compared to control (Fig. 7d). In line with these data, Ppargfl/fl Id2Cre+ mice showed a longer survival compared to control mice (Fig. 7e). To confirm our in vitro results on the involvement of ILC2s in the EMT phenomenon, we evaluated by qPCR analysis the expression of murine MMP-9 and N-cadherin in dissociated tumor tissues. As shown in Fig. 7f, higher expression of both Mmp9 and Ncad in Ppargfl/fl Id2Cre+ compared to Ppargfl/fl Id2Cre− tumors were observed. In addition, to evaluate the therapeutic potential of PPARγ inhibition in CRC development and progression, we treated tumor-bearing mice with T0070907. As shown in Supplementary Fig. 6a, treatment with T0070907 significantly reduced both tumor volume and weight, suggesting a possible therapeutic use of PPARγ inhibitors in CRC patients. Furthermore, we also assessed the pro-tumor effects of IL-13 in vivo by treating tumor-bearing mice with an anti-IL-13 neutralizing antibody. After 18 days of treatment, both tumor volume and weight were significantly reduced in treated mice compared to controls (Supplementary Fig. 6b). Taken together, these results underscore the relevance of ILC2s and PPARγ in fostering cancer cell progression and subsequent metastatic potential.

**Discussion**

ILC2s are emerging as key regulators of Type-2 immune responses, by influencing downstream adaptive immunity in
Lastly, by inducible and conditional deletion of PPARγ CRC, ILC2s show PPARγ affects the production of IL-5 and IL-13 in vivo53. However, the expression of PD1 on ILC2s is controlled by PPARγ. It has been derived 15d-PGJ2, as it was previously reported19. It has been found that IL-33 drives Th2 responses and the Th9 function is positively regulated by PPARγ, which promotes IL-9 secretion in acute allergic skin diseases49. Different ILC transcriptomic analysis previously reported on PPARγ mRNA expression in ILC2s, in both human and mouse tissues11. Moreover, it has been recently reported that PPARγ is an important regulator of ILC2 during allergic airway inflammation51,52. In addition, Batyrova et al., demonstrated that the expression of PD1 on ILC2s is controlled by PPARγ and affects the production of IL-5 and IL-13 in vivo53. However, the functional relevance of this nuclear receptor in ILC2s is still limited, particularly in humans. In contrast to the activation-induced PPARγ expression in CD4+ T cells22, our results argue for a steady-state expression of PPARγ in ILC2s, that apparently acts as an homeostatic regulator of Type-2 cytokine secretion, likely in the presence of endogenous ligands such as PGD2-derived 15d-PGJ2, as it was previously reported19. It has been demonstrated that PPARγ stimulation, via the induction of PGC-1α, promotes mitochondrial biogenesis in different cell types54. Our findings are consistent with a role of PPARγ in the homeostasis and disease. However, both extrinsic and cell-intrinsic regulatory pathways dictating ILC2 functions remain elusive. Here, we demonstrate that PPARγ is selectively expressed in ILC2s, both in humans and mice. Its pharmacological inhibition impairs mitochondrial fitness and reduces Type-2 cytokine secretion in ILC2s upon IL-33 stimulation. Furthermore, in the environment of a human IL-33-enriched cancer type, such as CRC, ILC2s show PPARγ-dependent pro-tumoral functions. Lastly, by inducible and conditional deletion of PPARγ in ILCs using PPARγ/Il2CreER<sup>12</sup> mice and a heterotypic tumor model, we show significant reduction of cancer development and progression in vivo. Overall, our results point to a PPARγ-driven pro-tumorigenic role of ILC2s in IL-33-dependent tumors and suggest that PPARγ targeting in ILC2s may become an attractive add-on therapy for ILC2-driven diseases, including cancer.

It has been reported that fatty acid metabolism-related genes (including hexokinase 2 (HK2), pyruvate dehydrogenase kinase 1 (PDK1), and FATP-6) play a key role in ILC2 functions during helminthic infections13. In other immune cell types, PPARγ was shown to act as a master regulator of lipid metabolism in alveolar macrophages46 as inhibitor of pro-inflammatory cytokine secretion47, and to promote type-2 responses48. In that regard, Nobs et al. found that IL-33 drives Th2 responses and the development of pulmonary allergic inflammation by induction of PPARγ in CD4<sup>+</sup> T cells and DCs22. More recently, it has been reported that Th9 function is positively regulated by PPARγ, which promotes IL-9 secretion in acute allergic skin diseases49. Different ILC transcriptomic analysis previously reported on PPARγ mRNA expression in ILC2s, in both human and mouse tissues11. Moreover, it has been recently reported that PPARγ is an important regulator of ILC2 during allergic airway inflammation51,52. In addition, Batyrova et al., demonstrated that the expression of PD1 on ILC2s is controlled by PPARγ and affects the production of IL-5 and IL-13 in vivo53. However, the functional relevance of this nuclear receptor in ILC2s is still limited, particularly in humans. In contrast to the activation-induced PPARγ expression in CD4<sup>+</sup> T cells22, our results argue for a steady-state expression of PPARγ in ILC2s, that apparently acts as an homeostatic regulator of Type-2 cytokine secretion, likely in the presence of endogenous ligands such as PGD2-derived 15d-PGJ2, as it was previously reported19. It has been demonstrated that PPARγ stimulation, via the induction of PGC-1α, promotes mitochondrial biogenesis in different cell types54. Our findings are consistent with a role of PPARγ in the...
maintenance of energetic homeostasis also in ILCs, possibly by boosting PGC-1α expression and promoting mitochondrial activity. Further, the expression of IL-13 and IL-5 could be directly regulated by the PPARγ-RXRα heterodimer in Type-2 cytokine epigenetically-poised ILC2s, as suggested by the presence of potential PPARγRXRα binding elements in the promoter regions of these genes. Whether or not PPARγ expression is heterogeneous in distinct developmental stages or in subpopulations of ILC2s across tissues remains to be elucidated by high-definition screening methods, such as single-cell mRNA sequencing.

Accumulating evidence has indicated that IL-33 and its receptor ST2 (also known as IL1RL1) represent a key inflammatory pathway in tumor biology and immunology, in particular, several studies reported that IL-33 and ST2 are implicated as potent modulators of the TME promoting immune cells recruitment and tumor malignancy in different types of cancer, including breast cancer, hepatocellular carcinoma, gastric cancer, and CRC. In CRC, different reports showed that IL-33 expression has been associated with good prognosis and the use of PPARγ agonists troglitazone and rosiglitazone inhibited tumorigenesis in CRC and bladder cancer models by a tumor-directed effect. On the other hand, in Apcmin mice the same treatment increases the number of tumors, and the use of PPARγ inhibitors (including T0070907) suppressed breast cancer cell proliferation in vitro and restrained CRC cell migration and invasion both in vitro and in vivo. In our setting of PPARγ targeting in ILC2s, the observed anti-tumoral phenotype obtained by the in vivo conditional and inducible genetic deletion of PPARγ in ILCs suggests that ILC-specific and temporal targeting of PPARγ might beneficial in
cancer. Thus, we identified PPARγ as a druggable target to suppress the potent and rapid ILC2 effector functions. Its pharmacologic inhibition might be used as an add-on therapy for cancer treatment and/or in other ILC2-mediated diseases, as recently demonstrated in allergy and asthma.22,48,73.

Methods
Human peripheral blood cell collection. Venous blood was drawn from healthy donors (HDs) at the Swiss Transfusion Center CRS of Lausanne. Volunteers are asked to read an information sheet for blood donation and to complete an online medical questionnaire on the day of donation. Once the questionnaire finalized, a PDF file is generated for printing, and it is signed to give the approval of the blood donation. PBMCs were isolated by Lymphoprep (Stemcell) centrifugation and immediately cryopreserved.

CRC patients. Donors of CRC tissues gave written informed agreement and analyses of the samples were approved by the Cantonal Ethics Committee of Bern (2017-01821 and 2018-01502). Leftover material from diagnostic was used for the tumor microarray (TMA), no extra tissue was collected specifically for this study. Metastatic colorectal cancer patients were included in the “Epitopes-CRC01” (NCT02838381) trial, blood samples were collected before any metastatic cancer specific treatment. For patients from “Epitopes-CRC01” (NCT02838381), tumor samples were excised by surgeons from the department of digestive surgery at University Hospital Bascon. The generation of TIL cultures was based on the methodology previously established by Dudley et al.74.

We have complied with all relevant ethical regulations for work with human participants, and we confirm that informed consent was obtained from each donor.

Flow cytometry analysis. Human ILC2s were identified as lineage (Lin) negative and CD127 positive cells. Lineage markers, all FITC-conjugated, include: anti-human CD3 (UCHT1, Beckman Coulter (BC) 1:50), anti-human CD4 (SFC117-1D11, BC, 2:50), anti-human CD8 (MEM-31, Immunotools, 1:50), anti-human CD14 (RM052, BC, 2:50), anti-human CD15 (80H5, BC, 2:50), anti-human CD16 (3G8, BC, 1:100), anti-human CD19 (J3-119, BC, 1:100), anti-human CD69 (H3-94, BC, 2:50), anti-human CD20 (2H7, Biologend, 1:100), anti-human CD3 (H12F1, BD Bioscience, 2:50), anti-human CD4 (361, Biologend, 1:100), anti-human CD203c (E-NPPS, 1:50) (N94D6, Biologend, 2:50), anti-human FeRγ (AER-37, Biologend, 2:50) anti-human CD6 (HEA-196, Miltenyi, 1:50). Additional markers used include: Brilliant Violet 421 anti-human anti-CD127 (IL-7Ra) (A019D5, Biologend, 1:100), APC anti-human CD117 (cKit) (YB5.84, BD Bioscience, 1:50), PE anti-human CRTH2 (CD294) (BM16, Biologend, 1:50). Dead cells were excluded using the viability dye Live/Dead Aqua or Vivid IR (Invitrogen). Representative gating strategy is included in Supplementary Fig. 7a. Intracellular staining was performed after fixation and permeabilization with 0.1% saponin (Sigma), using PE-Cy7 (JES10-5A2, Biologend, 1:50). Mmp9 (left) and Ncad (right) assessed by qPCR analysis in Pparγfl/flID2CreERT² positive (open blue circle) or negative (open red circle) dissociated tumor tissues (n = 8; Mmp9 * P = 0.0379, Ncad * P = 0.0104). Each symbol represents one individual mouse or sample. Data are shown as mean ± SEM and were analyzed by Log-rank (Mantel-Cox) (e, a) Wilcoxon (f) or two-way (b, d) ANOVA tests. Source data are provided as a Source data file.

Fig. 7 PPARγ expression in ILC2s influences cancer progression in a CRC murine model. a Tumor survival of RORγt/flID2Cre positive (blue bar) or negative (red bar) mice after implantation of MC-38-IL33 murine CRC cells (ILC2 WT n = 6, ILC2 KO n = 7; * P = 0.0227). b Representative image of tumor size (left) and tumor development (right) expressed as tumor volume in ILC2 WT (open red circle) and ILC2 KO mice (open blue circle) and ILC2 KO mice ILC2 transferred (open green circle) (n = 6; * P = 0.0473, ** P = 0.0023). c Schematic representation of CRC murine model in Pparγfl/flID2CreERT² mice. d Tumor development expressed as tumor volume in Pparγfl/flID2CreERT² positive (open blue circle) or negative mice (open red circle) (n = 15; * P = 0.0451). e Survival of Pparγfl/flID2CreERT² positive (blue bar) or negative (red bar) tumor-bearing mice (n = 5; * P = 0.0338). f Expression of Mmp9 (left) and Ncad (right) assessed by qPCR analysis in Pparγfl/flID2CreERT² positive (open blue circle) or negative (open red circle) dissociated tumor tissues (n = 8; Mmp9 * P = 0.0379, Ncad * P = 0.0104). Each symbol represents one individual mouse or sample. Data are shown as mean ± SEM and were analyzed by Log-rank (Mantel-Cox) (e, a) Wilcoxon (f) or two-way (b, d) ANOVA tests. Source data are provided as a Source data file.
antigen-CD4 (RPA-T4, Biologend, 1:50), Brilliant Violet 785 anti-human CD45RO (UCHL1, Biologend, 1:50), PE-Cy7 anti-human CXCR3 (1C8, Biologend, 2:50), PE anti-human CCR4 (RM16, Biologend, 1:50), and anti-human CD38 (CCR6) (G034E3, Biologend, 2:50). Cells were gated on alive CD3+ CD4+ CD45RO+ and the Th subset were sorted as: Th1 as CXCR3+ CTHL2+ CCR6+, Th2 as CTHL2+ and Th17 as CXCR3+ CTHL2+ CCR6+. 

Murine ILC2s were identified as CD45+ Lin- CCR6+ cells. Lineage markers, all FITC-conjugated, include: anti-mouse CD3 (17A2, in house, 1:200), CD5 (37.5, in house, 1:200), CD19 (ID3, in house, 1:200), CD11b (M1/70, in house, 1:200), CD11c (N418, in house, 1:200), B220 (RA3-6B2, in house, 1:200), CD49b (DX5, Miltenyi Biotec, 1:200), FCerIA (MAP-1, Miltenyi Biotec, 2:50), rTer119 (Ter119, in house, 1:200), CD16/32 (2.4G2, in house, 1:200) and TCIRG1 (Biolegend, 1:200). Additional markers used to identify the ILC subpopulations include: Alexa-Fluor 700 anti-mouse CD45.2 (A1-4A2, in house, 1:100), PE anti-mouse ST2 (RMST2-2, Invitrogen, 1:200), Brilliant Violet 605 anti-mouse CD90.2 (53.2-1, Biologend, 1:50), PE-Cy7 anti-mouse KLRG1 (2F1/KLRG1, Biologend, 1:100), APC anti-mouse CD117 (Kit) (2B8, ebioscience, 1:200), BV510 anti-mouse NP-K46 (29A1.4, Biologend, 1:100), BV711 anti-mouse NK1.1 (PK136, Biologend, 1:100), BV395 anti-mouse CD4 (GK1.5, BD, 1:200). 

**Peroxisome proliferator response element (PPRE) motif search in gene promoters.** The promoter sequences (~100 bp up- and downstream flanking regions) of human or mouse IL-5 and IL-13 were obtained from the Eukaryotic Promoter Database (human genome version Dec 2013 GRCh38, mouse genome version Mar 2012 mm10; https://epd.epfl.ch/index.php). The consensus sequences of the binding motif of the human or mouse PPARy-RXRα heterodimer were downloaded from the Jaspar2020 database (matrix IDs: MA0056.1, MA0065.2 (mouse), http://jaspar.generegnet.org/). Two methods were used to identify the presence of the PPRE. First, the findMotifs.pl method of the HOMER software

**Quantitative real-time PCR (qPCR)**. Total RNA was isolated from highly pure, sorted human ILC subsets using the TRIzol reagent according to the manufacturer's instructions (Invitrogen). Final preparation of RNA was considered DNA-free when the ratio of absorbance at 260/280 nm was ~2.1. Isolated mRNA was reverse-transcribed by the iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Milan, Italy). The quantitative real-time PCR was carried out in the Applied Biosystems 7900HT Fast Real-Time PCR Sequence Detection System (Applied Biosysytems) with specific primers (hPFPAR3 +5AGATTTGCTAGTCACTGCT-3’, 5’- GGCCAGCAACACTGGATTCC-3’; hPPARG +5AGGATGGAGGTTTCTCCTTC-3’, 5’- TCTAAGGTCCGCTTCTTTCT-3’; hMMP9 +5AGGAGACAAGCTGGAACAGTGC-3’, 5’- GATGCCGCAAGCAGATGAGACTTGC-3’; hCRTH2 +5AGGTGAGGTTGAAGGTGAAAG-3’, 5’- AGGCCCTCGTGCGTTGGCTTTGCAT-3’; hCCL2 +5AGGTGAGGTTGAAGGTGAAAG-3’, 5’- AGGCCCTCGTGCGTTGGCTTTGCAT-3’; hCCL5 +5AGGTGAGGTTGAAGGTGAAAG-3’, 5’- AGGCCCTCGTGCGTTGGCTTTGCAT-3’; hPPAR γ -5AGGTGAGGTTGAAGGTGAAAG-3’, 5’- AGGCCCTCGTGCGTTGGCTTTGCAT-3’). Samples were amplified simultaneously in triplicate in one-assay run with a non-template control blank for each primer pair to ensure contamination and primer dimerization, and the Ct value for each experimental group was determined. The housekeeping genes (ribosomal protein S16 and beta-2-microglobulin (B2M)) were tested to ensure normalization of the Ct values, using the 2-ΔΔCt formula. 

Alternatively, snap-frozen tissue and matched non-tumor tissue from CRC patients (n = 10 + 10) were homogenized using a TissueLyser (Qiagen) (3 x 30 s at 30 Hz). Total RNA was isolated following the protocol for TRI-reagent (Sigma-Aldrich) and eluted in 50 μl of water. Total RNA was then reverse-transcribed into cDNA using a Promega kit containing M-MLV-RT and Oligo(dT) primers, following the manufacturer’s protocol.

**Preparation of cellular extracts and western blot analysis.** Whole-cell extracts were prepared using RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM phe- nylmethylsulfonyl fluoride (PMSF), 1 mM Na3VO4, 5 mM NaF, and 1% cocktail protease inhibitors; Sigma). Total protein concentrations in cells after transfection were confirmed by qPCR analysis. The Universal scrambled negative control siRNA duplex was used as negative control.

**ChIP assay.** Expanded human ILC2s were cross-linked with 1% formaldehyde for 10 min, then washed for 3 times for 10 min in PBS, and proteins were visualized by the Pierce® BCA Protein Assay Kit (Thermo Scientific). Equal amounts of protein (40 μg/sample) were separated by electrophoresis in a 12% denatured polyacrylamide gel and blotted onto nitrocellulose membranes (Biorad). The membranes were blocked for 1 h in 5% low-fat milk in 1× PBS with 0.1% Tween20 (PBS) at room temperature. The filters were then incubated overnight at 4 °C with the following primary antibodies: PPARγ (2443, Cell Signaling, diluted 1:1000) and α-Tubulin (3873, Cell Signaling, diluted 1:1000). The membranes were washed 3 times for 10 min in PBS, and then incubated with HRP-conjugated anti-rabbit or anti-mouse antibodies (Biologend) for 2 h at room temperature. The membranes were then washed for 3 times for 10 min in PBS, and proteins were visualized by the ECL chemiluminescence method. The immunoreactive bands of proteins were quantified using ImageJ software.
Electron microscopy (EM). Sections were post-fixed in glutaraldehyde solution (EMS, Hatfield, PA, US) 2.5% in phosphate buffer (PB 0.1 M pH7.4) (Sigma, St Louis, MO, US) during 1 h at room temperature (RT). Then, they were directly post-fixed by a fresh mixture of osmium tetroxide 1% (EMS, Hatfield, PA, US) with 1.5% of potassium ferrocyanide (Sigma, St Louis, MO, US) in PB buffer during 1 h at RT. The samples were then washed three times in distilled water and dehydrated in acetone solution (Sigma, St Louis, MO, US) at graded concentrations (30%—40 min; 50%—40 min; 70%—40 min; 100%—3×1 h). This was followed by infiltration in Epon (Sigma, St Louis, MO, US) at graded concentrations (Epon 1/3 acetone-2h; Epon 3/1 acetone-2h, Epon 1/1-1 h Epon 1/1-12 h) and finally polymerized for 48 h at 60 °C in the oven. Ultrathin sections of 50 nm were cut on a Leica Ultracut (Leica Mikrosysteme GmbH, Vienna, Austria) and picked up on a copper slot grid polymer and Fast Red as red chromogen (Bond Marque, #390M-14; dilution of 1:400) primary antibodies. A rabbit anti-goat antibody (ab16669; dilution of 1:400) or anti-human IL-33 and anti-human GATA3 (Cell Biolegend). Briefly, antibodies specific for the 13 analytes were conjugated to 13 different fluorescence-encoded beads. The beads were mixed with the supernatants, incubated for 2 h at room temperature, washed, and incubated for 1 h with detection antibodies. Finally, streptavidin-PE was added and incubated for 30 min, and the beads were washed and acquired using Gallios. The results were analyzed by using the Legendplex software (version 6.0).

Multiplex cytokine assay. The concentrations of various cytokines in ILC2 supernatants, healthy donor’s and patient’s sera were determined using the multi-LEGENDplex™ analyte flow assay kit (human and mouse Th Panel (13-plex), Biolegend). Briefly, antibodies specific for the 13 analytes were conjugated to 13 different fluorescence-encoded beads. The beads were mixed with the supernatants, incubated for 2 h at room temperature, washed, and incubated for 1 h with detection antibodies. Finally, streptavidin-PE was added and incubated for 30 min, and the beads were washed and acquired using Gallios. The results were analyzed by using the Legendplex software (version 8.0).

ELISA. IL-33 plasma concentrations were evaluated using ELISA kit according to the manufacturer’s instruction (LEGEND MAX™ Human IL-33 ELISA Kit, Biolegend).

IHC. Human CRC tissues were fixed in 4% formaldehyde and embedded in paraffin. Staining reactions were performed by automated staining using a BOND RX autostainer (Leica Biosystems). For double immunohistochemistry staining, sections were first deparaffinized and antigen was retrieved using 1 mm Tris solution (pH 9.0) for 30 min at 95 °C. Sections were then stained with goat-anti-human ILC3 (R&D Systems, # AF6325; dilution of 1:400) and anti-human CD3 (abcam, # ab16669; dilution of 1:400) or anti-human IL-33 and anti-human GATA3 (Cell Marque, #390M-14; dilution of 1:400) primary antibodies. A rabbit anti-goat antibody (Agilent, # E0466) was used as secondary antibody, at a dilution of 1:400. Specific binding of primary antibodies was visualized using a polymer-based visualizing system with horseradish peroxidase as the enzyme and 3,3-diaminobenzidine (DAB) as a brown chromogen, or an alkaline phosphatase-linked polymer and Fast Red as red chromogen (Bond™ Polymer Reine and Red Detection, respectively) (all from Leica Biosystems). The samples were counterstained with hematoxylin and mounted with AquaTect (Merck). Slides were scanned in high resolution on whole slide scanners Pannoramic 250 Flash (3DHISTECH) or NanoZoomer S360 (Hamamatsu). All human CRC tissues were provided by the Tissue Bank Bern.

Image processing. Each MRI image was dearrayed and individual cores exported as PNG using QuPath.12 Color deconvolution was performed using ImageJ (Version 1.52p) and its “color deconvolution plugin” resulting in three individual channels for hematoxylin, IL33 and CD3 or GATA3, respectively. Since the CD3 and GATA3 stainings were performed on serial section, they could be co-registered using the ImageJ plugin “burwp” based on the hematoxylin channel.

In vitro co-culture experiments. Expanded ILC2s from healthy donors were pretreated with T0070907 PPARγ antagonist for 48 h and then cultured with the SW1116 polyclonal cell line in a ratio of 1:10 for 48 h, IL2C2 (ILC2), IL2C (ILC2 and IL7) or ILC2 + T0070907 CM (30% v/v) before adding 25 µL MTT (Sigma-Alrich; 5 mg/mL in saline). Cells were then incubated for additional 3 h at 37 °C. After this time interval, dark blue crystals were solubilized with pure DMSO. The optical density of each well was measured with a microplate spectrophotometer (TiterTrekMultis FCC340), equipped with a 540-nm filter.

Clonogenic assay. SW1116 cells (1 × 106 cells/well) were seeded in 6-well plates with IL2C2, IL2C + T0070907 CM (30% v/v) or ILC2 CM with anti-IL-13 blocking antibody (IL130). Cells were cultured for 14 days to allow the colonies to form. Formed colonies were washed twice with 1xPBS, fixed by 4% paraformaldehyde, and stained with 0.5% crystal violet and colonies containing more than 50 cells (established by microscopy) were counted manually. Images of the colonies were obtained using a digital camera. The experiments were done at least three times, in duplicate.

In vivo tumor models. PPARγfl/fl/IgG Tg mice were backcrossed to C57BL/6 mice and crossed in house with Id2-CreERT2 (Jackson Laboratory, Stock number 016222). To induce the deletion of PPARγ after intraperitoneal (i.p.) tamoxifen administration, ROARα/βCre/IgG/Cm mice were kindly provided by Prof. A. McKenzie.24 PPARγfl/fl/IId2-CreERT2 and ROARα/βCre/IgG/Cm mice and littermates, were bred between 6- and 12-weeks age and bred in house. All our mouse strains are in a C57BL/6 genetic background. 5 × 104 MC-38-IL33 murine colorectal cancer cells were injected subcutaneously (s.c.) in 200 µL of 1x PBS in the right flank and tumor growth was monitored. When indicated, T0070907 (7.5 mg/kg, Cayman Chemical), anti-IL-13 neutralizing antibodies (50 µg/mouse, InvivoGen) and Isotype controls (50 µg/mouse, Bscel) were daily injected intraperitoneally (i.p.) for 18 days. For ILC2 adoptive transfer, donor mice were injected with 0.4 µg of IL-25 and IL-33 for induction of ILC2s. 3 days after injection, flow cytometry-purified donor lung ILC2s were expanded in vitro (see Cell Culture) before 2.5 × 105 intravenous (i.v.) injection into ROARα/βCre/IgG/Cm mice. Tumor sizes were measured using a digital caliper and tumor volumes were calculated using the following equation: tumor volume = Π/6(D1 × D2 × D3) where D1 = length; D2 = width; D3 = height and expressed as cm3. We have complied with all relevant ethical regulations for animal testing and research. This study was approved by the Veterinary Authority of the Swiss Canton Vaud (authorization no. VD235S) and performed in accordance with Swiss ethical guidelines. All animals were maintained at the University of Lausanne’s animal facility under a 12 h dark/light cycle, at 21 °C ± 1 °C and 55% ± 10% HR.

Statistical analysis. Statistical analysis was performed using GraphPad Prism software version 6. For comparison of multiple groups ANOVAs or the non-parametric (Mann–Whitney or Kruskal–Wallis) tests were used. The data were shown by plotting individual data points and the mean ± SEM. A p value <0.05 (two-tailed) was considered statistically significant and labeled with *p values <0.01, 0.001 or 0.0001 were labeled with **, *** or ****, respectively.

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

Data availability. The RNA-seq data used in this study are deposited in the ArrayExpress under accession number E-MTAB-8494. Proteins putatively involved in protein–protein interactions were taken from the STRING database. The promoter sequences (~100 bp up- and downstream flanking regions) of human or mouse IL-5 and IL-13 were obtained from the Eukaryotic Promoter Database (human genome version Mar 2012 mm10; https://epd.epfl.ch/index.php). The consensus sequences of the binding motif of the human or mouse PPARγ RXRA heterodimer were downloaded from the Jaspar2020 database (matrix IDs: MA0065.1 (human), MA0065.2 (mouse), http://jaspar.genereg.net/). Source data are available as a Source data file. The remaining data are available within the Article, Supplementary Information or available from the authors upon request.
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