Innate lymphoid cells lacking surface expression of CD90 are functional

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Conflict of interest statement

The authors have declared that no conflict of interest exists.

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ABSTRACT

Huge progress has been made in understanding the biology of innate lymphoid cells (ILC) by adopting several well-known concepts of T cell biology. As such flow cytometry gating strategies and markers, such as CD90, to identify ILC were discovered. Here we report that most non-NK intestinal ILC have a high expression of CD90 as expected, but surprisingly some have only a low or even no expression of this marker. CD90-negative CD127⁺ ILC were identified among all ILC subsets in the gut. CD90-negative cLP ILC2 were frequent at steady state. The frequency of CD90-negative CD127⁺ ILC was dependent on stimulatory cues *in vitro* and *in vivo*, and CD90-negative CD127⁺ ILC played a functional role as a source of IL-13, IFN γ and IL-17A at steady state and upon dextran sulphate sodium-elicited colitis. Hence, this study highlights for the first time that CD90 is not constitutively expressed by functional ILC in the gut.

INTRODUCTION

Resident leukocytes play an important role in maintaining mucosal surfaces at steady state and early during an infection (Bal *et al.*, 2020, Klose *et al.*, 2020). Since the discovery of innate lymphoid cells (ILC) about a decade ago, it has been increasingly apparent that these cells play a significant role in mucosal homeostasis. However, their role is far from being fully characterised, and much of the current knowledge has been gained from testing concepts that had already been established for T and NK cell biology. As such, group 1, 2 and 3 ILC (ILC1, ILC2 and ILC3) express T-bet, GATA3 and ROR γ t respectively as characteristic transcription factors as well as cytokines relating to Th1, Th2 and Th17 cells (Bal *et al.*, 2020). Due to the absence of TCR expression in ILC, these cells elicit immune functions in response to cytokines, chemokines and neurotransmitters as has been well described for NK cells (Bal *et al.*, 2020, Klose *et al.*, 2020).

Similarly to T and NK cells, ILC express the glycoposphatidylinositol (GPI) anchored protein CD90 (Sauzay *et al.*, 2019), which has been used as key target to deplete ILC in Rag-deficient mice using a specific antibody (e.g. Powell *et al.*, 2012, Martin *et al.*, 2017, Rafei-Shamsabadi *et al.*, 2018, Castro-Dopico *et al.*, 2020). Despite its expression on T and NK cells very little is understood regarding its functionality (Sauzay *et al.*, 2019). Known ligands are integrins α v β 3, α x β 2, syndecan-4, CD97 and CD90 itself (Wandel *et al.*, 2012, Kong *et al.*, 2013 and Leyton *et al.*, 2014). *In vitro* studies in T cells suggested that CD90 activation with a specific antibody can promote proliferation as well as IFN γ , IL-17A and IL-13 production in unpolarized and polarized CD4⁺ T cells in particular in case of co-stimulation with an agonistic anti-CD28 antibody (Haeryfar *et al.*, 2004, Furlong *et al.*, 2018). In terms of ILC2, it has been observed that inflammatory ILC2 in

lungs and small intestinal lamina propria (SI LP) have a lower expression of CD90 in comparison to natural ILC2, but the implications for this have not been established (Huang *et al.*, 2014, Flamar *et al.*, 2020). In relation to this, CD90^{low} to CD90^{high} ILC2 precursor transition has been described using an *in vitro* model in which CLP were seeded, but again its role is unknown (Seehus *et al.*, 2014).

Here we report for the first time that colonic lamina propria (cLP) ILC have a differential expression of CD90, and strikingly some ILC show no expression of this marker. These CD90⁻ ILC are a significant source of IFN γ , IL-13, IL-5 and IL-17A upon dextran sulphate sodium (DSS)-elicited colitis. However, in naïve mice, CD90⁻ ILC have a dominant type 2 cytokine expression profile. Furthermore, stimulation with IL-25/IL-33 promotes the frequency of CD90⁻ ILC2 *in vitro*. Conversely, IL-12/IL-18 stimulation results in a lower prevalence of CD90⁻ NKp46⁺ ILC. These data suggest that CD90 expression in intestinal ILC is regulated by cytokines rather being a constitutive marker of the ILC lineage.

RESULTS

CD90-negative CD127⁺ ILC have a predominant type 2 phenotype at steady state

Analyses of Lin⁻ CD45⁺ (CD3, CD5, CD45R, CD19, CD11b, TER-119, Gr-1, FcεRI) CD127⁺ cLP ILC stimulated with PMA and ionomycin (PI) revealed that in addition to CD90^{high} ILC there were two additional CD90⁻ and CD90^{low} ILC populations (Figure 1a). The abundance of CD90^{high} ILC was greater than CD90⁻ and CD90^{low} ILC, but these populations represented ~ 5 % each from the total CD127⁺ ILC population. In order to associate CD90⁻ and CD90^{low} ILC with a specific ILC subset we analysed CD90⁻ and CD90^{low} ILC in *Tbx21*^{-/-} mice. This revealed that the presence of these cells was not dependent on this transcription factor, and their frequency was mostly unaffected with an only minor but significantly enhanced cellularity of CD90^{low} ILC. Interestingly, both CD90⁻ and CD90^{low} ILC expressed predominately IL-13 and IL-5 and fewer of these cells expressed IFNγ and IL-17A (Figure 1b). Although moderately low, CD90⁻ and CD90^{low} ILC had a significantly greater IFNγ expression frequency than CD90^{high} ILC (Figure 1c). A similar trend was not observed in terms of IL-17A expression (Figure 1d). IFNγ and IL-17A expression was also driven mostly by separate cells (Figure 1b). Further analyses revealed that the prevalence of IL-13⁺ and IL-5⁺ ILC was greater among CD90^{high} and CD90^{low} ILC in comparison to CD90⁻ ILC (Figure 1b, e, f, Supplementary Figure 1a). *Tbx21*^{-/-} CD90⁻, CD90^{low} and CD90^{high} ILC had a greater expression of IL-5 than WT mice which could be explained by one of our previous reports indicating that cLP ILC2 abundance is greater in *Tbx21*^{-/-} mice (Garrido Mesa *et al.*, 2019). Since CD90⁻ and CD90^{low} ILC appeared to be predominately functional ILC2 we sought to determine whether these cells are able to adopt functional characteristics of ILC1 and ILC3. Plasticity of ILC2 in order to express T-bet and RORγt are well-known phenomena (Bal

et al., 2020) and inflammatory ILC2 expressing ROR γ t were reported to have no or a lower expression of CD90 in comparison to natural ROR γ t-negative ILC2 (Huang *et al.*, 2015, Flamar *et al.*, 2020). We detected minimal co-expression of IL-13 and IL-17A in CD90⁻ and CD90^{low} cLP ILC from naïve WT and *Tbx21*^{-/-} mice indicating the presence of minor inflammatory ILC2 population (Supplementary Figure 1b). However, we could also find IL-13 and IL-17A co-expressing CD90^{high} ILC. In contrast, virtually no IL-13 and IFN γ co-expressing CD90⁻ and CD90^{low} ILC were detected in these mice (Supplementary Figure 1c). Despite the prevalence of ILC2 among CD90⁻ cLP ILC, we could also detect a significant population of KLRG1⁻ CD90⁻ cLP ILC, which are not ILC2 (Figure 1g). For these analyses KLRG1 was used as a key marker of intestinal ILC2 in line with recent publications (Flamar *et al.*, 2020, Garrido Mesa *et al.*, 2019, Schroeder *et al.*, 2021a). KLRG1 as a marker for intestinal ILC2 has an advantage to GATA3 as intestinal ILC3 have a low expression of GATA3 and the expression of this transcription factor is variable among the ILC2 population (Zhong *et al.*, 2016, Zhong *et al.*, 2020, Ricardo-Gonzalez *et al.*, 2018). KLRG1^{hi} intestinal ILC as gated in this study require GATA3 for post-developmental maintenance supporting the notion these cells are ILC2 (Yagi *et al.*, 2014).

Furthermore, we seeded bone marrow-derived ILC2 precursors (ILC2p) defined as Lin⁻ CD127⁺ α 4 β 7^{hi} Flt3⁻ CD25⁺ in a 6 day culture on OP9-DL1 stromal cells in the presence of rIL-7, rSCF and rIL-33 using an established method to develop ILC2 (Seehus *et al.*, 2016). Strikingly, the cell population generated also had a variable expression of CD90 (Figure 1h). Most of these ILC were CD90^{high}, but there were also substantial CD90⁻ and CD90^{low} subpopulations.

CD90 expression in CD127⁺ ILC is controlled by stimulatory cues

We next sought to determine whether CD90 expression can be altered by immunological stimulation. For this, we FACS sorted KLRG1⁺ cLP ILC2 and KLRG1⁻ cLP ILC for *in vitro* culture in the presence of distinct cytokines. Strikingly, ILC2 stimulation with IL-25 and IL-33 induced the presence of CD90^{-/low} ILC2 (Figure 2a, b). A similar but less potent effect was observed when IL-12 and IL-18 had been added to the culture medium. Additional IL-6/IL-1 β /TNF α /IL-27 stimulation did not further alter IL-12/IL-18-mediated CD90^{-/low} ILC2 frequency, while IL-1 β /IL-23 stimulation caused no alteration as well. Conversely to ILC2, IL-12/IL-18 stimulation of non-ILC2 in the presence or absence of IL-6/IL-1 β /TNF α /IL-27 resulted in fewer CD90^{-/low} NKp46⁺ ILC (Figure 2a, b). IL-1 β /IL-23 and IL-25/IL-33 stimulation of these cells had no effect in terms of CD90 expression. Stimulation with PMA and ionomycin or a soluble agonistic anti-CD28 antibody did also alter the frequency of CD90 expression levels in neither ILC2 nor non-ILC2. CD28 had been chosen as a stimulation target due to reports indicating the expression of CD28 in human ILC (Roan *et al.*, 2016, Björklund *et al.*, 2016).

CD90-negative cLP CD127⁺ ILC produce cytokines upon induced colitis

CD90⁻ and CD90^{low} ILC *in vivo* functionality was tested in a model of DSS-induced colitis. C57BL/6 mice were treated with 3% DSS in the drinking water for 5 days after which the animals showed clinical signs of colitis like weight loss (Schroeder *et al.*, 2021b), and the cytokine expression profile of cLP ILC was analysed at day 10. Similar to naïve mice (Figure 1), most ILC were CD90^{high}, however, CD90⁻ and CD90^{low} ILC populations were detected again, but at a lower prevalence (Figure 3a, b). These results

were also observed when DSS-treated BALB/c mice were used instead of C57BL/6 mice (Supplementary Figure 2a, b). Within these populations, IFN γ , IL-13 and IL-17A producing cells could be detected but unlike in naïve C57BL/6 mice, these cells did not appear as potent in producing IL-13 in DSS-treated C57BL/6 mice (Figure 1, Figure 3c). In these DSS-treated mice CD90^{high} ILC were a vastly more significant source of IFN γ , IL-13 and IL-17A in comparison to CD90⁻ and CD90^{low} ILC (Figure 3d). This may suggest that inflammatory cues driven by DSS are able to promote CD90^{high} ILC activation via IL-12 and IL-18, as suggested from the *in vitro* studies (Figure 2). In line with the data from naïve mice (Figure 1), T-bet deficiency did not promote the frequency of IL-13 or IL-17A producing cells among the ILC population. In contrast to C57BL/6 mice, CD90⁻ and CD90^{low} ILC were potent producer of IFN γ , IL-13 and IL-17A in DSS-treated BALB/c mice (Supplementary Figure 2c, d). A further difference to C57BL/6 mice was the increased frequency of IL-17A and IL-13 producing CD90⁻ and CD90^{low} ILC and IL-17A⁺ CD90^{high} ILC in DSS-treated *Tbx21*^{-/-} mice (Supplementary Figure 2c, d). We also detected CTLA4 expression among CD90^{high}, CD90⁻ and CD90^{low} ILC in DSS-treated C57BL/6 and BALB/c mice (Figure 3c, d, Supplementary Figure 2c, d). This is of interest since expression of this molecule has been associated with inflammatory ILC2 (Nagashima *et al.*, 2019, Ricardo-Gonzalez *et al.*, 2018, Flamar *et al.*, 2020). To address potential ILC2 plasticity, we detected no IFN γ producing IL-13⁺ ILC, but IL-17A production among CD90⁺ and CD90⁻ IL-13⁺ ILC2 (Figure 3e). These data indicate that low expression of CD90 cannot be associated with all inflammatory ILC2 in these mice.

In order to validate these data, cLP ILC were analysed in *Rag2*^{-/-} and *Rag2*^{-/-} *Tbx21*^{-/-} (TRnUC) mice upon DSS-induced colitis. BALB/c background mice were used on this occasion as these develop greater colitis compared to mice on a C57BL/6 background

(Ermann *et al.*, 2001). CD90⁻ and CD90^{low} ILC were again relevant sources of IFN γ , IL-13 and IL-17A, but still significantly less potent than CD90^{high} ILC in these DSS-treated *Rag2*-deficient mice (Figure 4c, d). DSS-treated TRnUC mice did not have altered frequencies of CD90⁻ and CD90^{low} ILC or IL-13 expression in these cells in comparison to DSS-treated *Rag2*^{-/-} mice (Figure 4b-d). However, these TRnUC mice had a greater frequency of IL-17A expressing CD90⁻ and CD90^{high} ILC than the respective *Rag2*^{-/-} ILC. This could be explained by the far greater cellularity of ILC3 in *Rag2*^{-/-} mice driven by the deficiency of *Tbx21* (Schroeder *et al.*, 2021a). Similar to the observations in DSS-treated WT mice, we detected CTLA4 expression among CD90^{high}, CD90⁻ and CD90^{low} ILC and CD90⁻ and CD90⁺ inflammatory ILC2 co-expressing IL-13 and IL-17A (Figure 4c, d, e, f). We also detected more CD90⁺ than CD90⁻ inflammatory ILC2 (Figure 4e, f) further supporting the notion that inflammatory ILC2 appear with a CD90⁻ and CD90⁺ phenotype. We also noticed that CD90⁻ ILC can also express IL-17A independently of IL-13 (Figure 4f). Interestingly, T-bet-deficiency appears to promote the frequency of CD90⁻ IL-17⁺ among IL-13⁺ ILC2 in these *Rag2*^{-/-} mice.

Overall, the data in this study suggest that CD90 is not a reliable marker to detect all ILC in the gut. When we analysed CD127 and CD90 co-expression in lineage-negative cLP leukocytes, we noticed that almost all CD90⁺ cLP ILC had a detectable surface expression of CD127 in naïve C57BL/6 WT and DSS-treated C57BL/6 WT, BALB/c WT and BALB/c *Rag2*^{-/-} mice (Supplementary Figure 3).

DISCUSSION

Ever since the discovery of ILC about a decade ago, there have been refinements to the ILC analysis strategy by flow cytometry. This is still an active process, as an increasing amount of functional states within the 3 ILC groups are being reported. In the past many groups have used CD90 as a pan marker for ILC and CD90-specific antibodies are often employed to deplete ILC *in vivo* (e.g. Powell *et al.*, 2012, Martin *et al.*, 2017, Rafei-Shamsabadi *et al.*, 2018, Castro-Dopico *et al.*, 2020). However, we suggest the suitability of CD90 to detect ILC has limitations, especially when analysing intestinal ILC. In contrast to the notion that CD90 is a pan ILC marker, the data presented in this study outline that intestinal ILC can be separated into CD90⁺ and CD90^{high} ILC in addition to CD90^{low} ILC, which are most likely transitional cells. In our hands CD127 is a far more reliable marker to detect ILC than CD90. Virtually all CD90⁺ cLP ILC express CD127, however, other reports indicate that pulmonary ILC can lose CD127 *in vivo* and IL-7 downregulates CD127 expression in ILC *in vitro* (Cavagnero *et al.*, 2019, Martin *et al.*, 2017). Hence, in the absence of better ILC markers we would advise using a combination of CD127 and CD90 to detect ILC.

In BALB/c mice, CD90⁻ ILC account for about a fifth to a third of cLP ILC, and we detected a substantial amount of IFN γ , IL-13 and IL-17A produced by these cells upon treatment with DSS. Hence, we believe these data implicate that these cells play a relevant role in the ILC response in intestinal tissue. It is out of the scope of this report to define a functionality of CD90 for ILCs, but it was striking to notice that while most cytokine-producing cLP CD90⁻ ILC were ILC2 in C57BL/6 at steady state, lack of CD90 expression was not restricted to ILC2. The cytokine combination of IL-33 and IL-25,

known to activate ILC2, was a potent stimulus to downregulate CD90 in cLP ILC2 *in vitro* suggesting that low CD90 expression may be an indicator for intestinal ILC2 activity. Interestingly, CD90 expression in pulmonary ILC2 was also shown to drop upon stimulation with IL-33 (Entwistle *et al.*, 2020). cLP ILC2 stimulation with IL-12 and IL-18 also enhanced the frequency of CD90^{low} cells. It has been reported that IL-12/IL-18 and IL-25/IL-33 can induce ILC2 to express T-bet and RORγt respectively (Silver *et al.*, 2016, Flamar *et al.*, 2020). In a model of DSS-induced colitis we could not associate either IFNγ or IL-17A production by cLP ILC2 with loss of CD90 expression. In contrast to ILC2, CD90 expression was enhanced by IL-12/IL-18-mediated stimulation in NKp46⁺ cLP ILC, which may further indicate that CD90 plays a functional role. Interestingly, CD90⁻ and CD90^{low} ILC expressed CTLA4 upon DSS-elicited colitis, and this marker is upregulated in activated ILC. CD90 was demonstrated to regulate PPARγ expression in adipocytes (Woeller *et al.*, 2015), and other groups have reported previously that PPARγ plays an important role for ILC2 functionality outlining one hypothetical role of CD90 (Karagiannis *et al.*, 2020, Xiao *et al.*, 2021).

The functional role of CD90 expression on ILC is unknown and is ill-defined as for other lymphocytes. In terms of T cells, it has been revealed that CD90 and CD28 co-stimulation can trigger T cell activation, but further work is required to determine the significance of this signalling axis (Haeryfar *et al.*, 2004, Furlong *et al.*, 2018). Due to the numerous known ligands several intercellular interactions of ILC with T cells, NK cell, neuron, glial cells, fibroblasts or endothelial cells may occur (Sauzay *et al.*, 2019). We suggest the data presented in this study outline that intestinal ILC can be separated into CD90⁺ and CD90⁻ ILC and believe these data have critical implications to understand ILC functionality in intestinal tissue.

METHODS

Animals

C57BL/6 WT, *Tbx21*^{-/-} (both C57BL/6 and BALB/c background) and *Rag2*^{-/-} (BALB/c background) mice were sourced commercially (Charles River). A colony of colitis-free BALB/c *Rag2*^{-/-} x *Tbx21*^{-/-} (TRnUC) mice was generated from a descendant of the TRUC colony described previously (Garrett *et al.*, 2007, Powell *et al.*, 2012, Powell *et al.*, 2015). All mice were housed in specific pathogen-free facilities at King's College London Biological Services Unit or at Charles River Laboratories.

Isolation of cells

cLP leukocytes were isolated using a published method (Grohnke *et al.*, 2017). Briefly, the epithelium was removed by incubation in HBSS lacking Mg²⁺ or Ca²⁺ (Invitrogen) supplemented with EDTA and HEPES. The tissue was further digested in 2% of foetal calf serum (FCS Gold, PAA Laboratories) supplemented in 0.5 mg/ml collagenase D, 10 µg/ml DNase I and 1.5 mg/ml dispase II (all Roche). The LP lymphocyte-enriched population was harvested from a 40%-80% Percoll (GE Healthcare) gradient.

Flow Cytometry

Flow cytometry was performed using a standard protocol. Fc receptor blocking was carried out with anti-CD16/32 specific antibodies. A lineage cocktail of antibodies specific for CD3, CD45R, CD19, CD11b, TER-119, Gr-1, CD5 and FcεRI was used for cLP ILC analyses. Live/Dead Fixable Blue Cell Stain Kit (Invitrogen) stain was used to exclude dead cells from the analysis. The cLP ILC gating strategy is outlined in our recent publications (Schroeder *et al.*, 2021a, Schroeder *et al.*, 2021b). For a complete list of the

antibodies used see Table 1. A FoxP3 staining kit (ebioscience) was used for intracellular staining of cytokines and CTLA4. In case of cytokine and CTLA4 expression analyses, cells were pre-stimulated with 100 ng/ml PMA and 2 μ M ionomycin in the presence of 6 μ M monensin for 3-4 hours prior to flow cytometry analysis. Samples were acquired using an LSRFortessa™ cell analyser (Becton Dickinson, USA), and all the data were analysed using FlowJo software (Tree Star, USA).

ILC2 generation in OP9-DL1 system

ILC2p were seeded on OP9-DL1 to generate ILC2 using an established method (Seehus *et al.*, 2016). Briefly, 7,500 cells were co-cultured with mitomycin pre-treated OP9-DL1 in presence of rmIL-7, rmSCF and rmIL-33 (all 20 ng/ml) for 6 days prior to FACS analysis.

cLP ILC Sorting and *in vitro* culture

Single-cell suspensions from colonic lamina propria were stained with fluorescently labelled antibodies and isolated using a BD FACSAria III cell sorter (BD Biosciences). Live CD45⁺ Lin⁻ CD127⁺ ILC FACS sorted as KLRG1⁺ and KLRG1⁻ were cultured in DMEM supplemented with 10% FCS, 1xGlutaMax (Gibco), 50 U/ml penicillin, 50 μ g/ml streptomycin, 10 mM HEPES, 1x non-essential amino acids (Gibco), 1 mM sodium pyruvate and 50 μ M β -mercaptoethanol (Gibco). 20,000 cells were plated per well of a 96-well plate pre-seeded with OP9-DL1 using an established method (Seehus *et al.*, 2016, Schroeder *et al.*, 2013). The medium was further supplemented with rmIL-7 and rhIL-2 (both at 10 μ g/ml) and further recombinant mouse cytokines or anti-CD28 antibody (2 μ g/ml; clone 37.51) as indicated (all cytokines were used at a final concentration of 10 μ g/ml unless indicated otherwise). Cells were harvested and

analysed by flow cytometry after 1 day in culture. FACS sort-derived cells from these conditions were harvested and analysed without additional pre-stimulation.

***In vivo* models**

Colitis was induced by adding 3 or 5% DSS (36-50 KDa, MP Biomedicals, Ontario, USA) to the drinking water for 5 days and mice were sacrificed 5-10 days after the beginning of the treatment. To establish a non-colitic control condition, mice were administered sterile drinking water. Regarding all *in vivo* models, body weights and clinical abnormalities were monitored on a daily basis.

Statistics

Results are expressed as mean $\pm$ SEM. Data were analysed using Student's t-test using GraphPad Prism 5.0 (GraphPad Inc., USA). ns: non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Study approval

All animal experiments were performed in accredited facilities in accordance with the UK Animals (Scientific Procedures) Act 1986 (Home Office Licence Numbers PPL: 70/6792, 70/8127 and 70/7869).

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AUTHOR CONTRIBUTIONS

Study concept and design (JHS, GML, JFN), acquisition of data (JHS, TZ), data analysis and interpretation (JHS, GML, JFN), obtained funding (GML, JFN), drafting of manuscript (JHS), study supervision (GML).

DISCLOSURE

The authors have no conflict of interest to declare.

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FIGURE LEGENDS

Figure 1. Intestinal CD90-negative CD127⁺ ILC have predominant type 2 phenotype

cLP CD127⁺ ILC were isolated from untreated WT and *Tbx21*^{-/-} mice and stimulated with PMA and ionomycin (4 hours) prior to flow cytometry analysis. (a) Frequencies of CD90^{hi}, CD90^{low} and CD90⁻ in CD127⁺ ILC and statistical analyses are outlined (n=3). (b) IL-13, IL-5, IFN γ and IL-17A expression in CD90^{hi}, CD90^{low} and CD90⁻ total CD127⁺ ILC and statistical analyses of (c) IFN γ and (d) IL-17A, (e) IL-13 and (f) IL-5 expression flow cytometry analyses in CD90^{hi}, CD90^{low} and CD90⁻ CD127⁺ ILC are illustrated (n=3). (g) KLRG1 and CD90 co-expression in cLP CD127⁺ ILC was demonstrated by flow cytometry (n=12). (h) ILC2 were generated from ILC2p stimulated with rIL-7, rSCF and rIL-33, and seeded onto OP9-DL1. CD90^{hi}, CD90^{low} and CD90⁻ ILC2 are shown.

Figure 2. CD90-negative cLP ILC2 prevalence depends on stimulation with cytokines

KLRG1⁺ or KLRG1⁻ CD127⁺ ILC were isolated and stimulated *in vitro* for 48 hours prior to harvest and flow cytometry analyses of KLRG1⁺ or NKp46⁺ ILC, respectively. In addition to a control condition, soluble agonistic anti-CD28 antibodies, IL-12&IL-18, IL-1 β &IL23, IL-25&IL-33 or IL-12&IL-18& IL-1 β &IL-6&TNF α &IL-27 were used as stimuli. In a separate condition sorted cells were stimulated with PMA and ionomycin in the presence of monensin for the final 4 hours prior to harvesting. (a) Flow cytometry analyses of CD90^{hi} and CD90^{low/neg} CD127⁺ ILC and (b) statistical analyses of CD90^{low/neg} ILC frequencies among KLRG1⁺ or NKp46⁺ cLP ILC are outlined (n=3).

Figure 3. CD90-negative WT cLP CD127⁺ ILC are a minor source of IFN γ , IL-13 and IL-17A during DSS colitis

cLP ILC from 3% DSS-treated C57BL/6 WT and *Tbx21*^{-/-} mice were isolated and stimulated with PMA and ionomycin (3 hours) prior to flow cytometry analysis. (a) Frequencies of CD90^{hi}, CD90^{low} and CD90⁻ in total CD127⁺ ILC and (b) statistical analyses are outlined. (c) IFN γ , IL-13 and IL-17A and CTLA4 expression in CD90^{hi}, CD90^{low} and CD90⁻ CD127⁺ ILC and (d) corresponding statistical analyses are shown. (e) CD90 co-expression with IL-17A or IFN γ in IL-13⁺ ILC is demonstrated. Data shown are representative of 4 biological replicates.

Figure 4. CD90-negative Rag-deficient ILC are a substantial source of IFN γ and IL-13 during DSS colitis

cLP ILC from 5% DSS-treated *Rag2*^{-/-} and TRnUC mice were isolated and stimulated with PMA and ionomycin (3 hours) prior to flow cytometry analysis. (a) Frequencies of CD90^{hi}, CD90^{low} and CD90⁻ in total CD127⁺ ILC and (b) statistical analyses are shown. (c) IFN γ , IL-13 and IL-17A and CTLA4 expression in CD90^{hi}, CD90^{low} and CD90⁻ total CD127⁺ ILC and (d) corresponding statistical analyses are outlined. (e) CD90 co-expression with IL-17A or IFN γ in IL-13⁺ ILC and corresponding statistical analyses are shown. (f) Flow cytometry and statistical analysis of CD90 and IL-13 expression in IL-17A⁺ ILC are presented. Data shown are representative of 4 biological replicates.

Supplementary Figure 1: Prevalence of inflammatory ILC2 in untreated mice.

cLP CD127⁺ ILC were isolated from untreated WT and *Tbx21*^{-/-} mice and stimulated with PMA and ionomycin (4 hours) prior to flow cytometry analysis. (a) Statistical analysis of IL-5⁺ IL-13⁻ CD90^{hi}, CD90^{low} and CD90⁻ CD127⁺ ILC based on the flow cytometry analyses illustrated in Figure 1b (n=3). Flow cytometry analyses of IL-13 co-expression with (b) IL-17A and (c) IFN γ in CD90, CD90^{low} and CD90⁻ CD127⁺ ILC are outlined.

Supplementary Figure 2: CD90-negative WT cLP CD127⁺ ILC are a source of IFN γ , IL-13 and IL-17A upon DSS treatment of BALB/c mice

cLP ILC from 3% DSS-treated BALB/c WT and *Tbx21*^{-/-} mice were isolated and stimulated with PMA and ionomycin (3 hours) prior to flow cytometry analysis. (a) Frequencies of CD90^{hi}, CD90^{low} and CD90⁻ in total CD127⁺ ILC and (b) statistical analyses are outlined. (c) IFN γ , IL-13 and IL-17A and CTLA4 expression in CD90^{hi}, CD90^{low} and CD90⁻ CD127⁺ ILC and (d) corresponding statistical analyses are shown.

Supplementary Figure 3: CD127 is a more reliable marker for cLP ILC than CD90

cLP leukocytes were isolated from untreated C57BL/6 and DSS-treated C57BL/6, BALB/c and Rag-deficient BALB/c mice. CD127 and CD90 co-expression in lineage-negative leukocytes are shown.

Antibody	Clone	Company
$\alpha 4\beta 7$	DATK32	eBioscience
CD25	PC61.5	eBioscience
CD3	17A2	eBioscience
CD5	53-7.3	eBioscience
CD19	1D3	eBioscience
B220	RA3-6B2	eBioscience
CD11b	M1/70	eBioscience/Biolegend
Gr-1	RB6-8C5	eBioscience
Flt3	A2F10	eBioscience
Ter119	TER-119	eBioscience
Fc ϵ RI	MAR	eBioscience
CD127	A7R34	eBioscience
NKp46	29A1.4	eBioscience
IL-13	eBio13A	eBioscience
IFN γ	XMG1.2	eBioscience
CD45	30-F11	Invitrogen
CD90.2	29A1.4	eBioscience
IL-5	TRFK5	BD
IL-17A	PAJ-17R	eBioscience
KLRG1	2F1	eBioscience

Table 1: Antibody clones and distributors

Figure 1

Figure 2

A

B

Figure 3

Figure 4