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The long non-coding RNA *Dreg1* is required for optimal ILC2 development

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eLife Assessment

This is a **valuable** study that investigates the role of the long non-coding RNA Dreg1 for the development, differentiation, or maintenance of group 2 ILC (ILC2). The authors generate Dreg1-/- mice and show **solid** evidence for a reduction of group 2 innate lymphoid cells (ILC2). However, the strength of evidence supporting and analysing the impact of Dreg1 on Gata3 expression, a transcription factor required for ILC2 cell fate decisions, remains **incomplete**. This study will be of interest to immunologists.

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Abstract

Gata3 is an essential transcription factor for the development of several distinct immune cell lineages such as T cells, natural killer (NK) cells and innate lymphoid cells (ILC). As such, the levels and timing of *Gata3* expression are critical for directing lineage fate decisions. The *Gata3* locus has a complex and dynamic distal regulatory enhancer landscape. Recently we identified a non-coding RNA, *Dreg1*, located immediately upstream of the classic +280kb T/NK cell enhancer (Tce1). To test its function, we excised the *Dreg1* locus in mice and observed a selective reduction of group 2 ILCs (ILC2) across multiple tissues, but mature T, NK and other ILC lineages remained unchanged. In bone marrow, common innate lymphoid cell progenitors (ILCP) increased while ILC2 progenitors (ILC2P) decreased, with a modest reduction of *Gata3* in upstream progenitors consistent with an early developmental bottleneck. Chromatin profiling showed the Dreg1 locus is accessible in early lymphoid progenitors and became decorated with H3K27ac in ILCP in a Tcf1-dependent manner. Furthermore, Tcf1-deficient cells did not express *Dreg1* and showed alterations in the epigenetic landscape of the *Dreg1* locus. Finally, we discovered that potential homologues of *Dreg1* harboured in a syntenic enhancer of *GATA3* are also highly expressed in human ILC2. Taken together we conclude that *Dreg1* is a Tcf1-dependent non-coding RNA critical for fine tuning the high level of *Gata3* required for the optimal development of the ILC2 lineage.

Introduction

The immune system comprises a collection of functionally distinct cell types which act in concert to protect the body from infection and malignancy. These diverse cell populations develop from common haematopoietic progenitors in a highly controlled fashion driven by a network of transcription factors that control lineage-specific gene expression programs. GATA-binding protein 3 (Gata3) is one of the master transcription factors that regulates the development of immune cells such as T cells, Natural Killer (NK) cells, and innate lymphoid cells (ILCs) (1, 2). Gata3 is also critical for the development of $CD4^+$ T helper 2 (Th2) cells (3) which produce cytokines such as IL4, 5 and 13 that are critical for protection against extracellular pathogens. It has recently emerged that a population of ILCs known as group 2 ILCs (ILC2) with similar characteristics to Th2 cells reside in non-lymphoid tissues such as the small intestine, visceral adipose tissue and lung and have been

shown to play a non-redundant role in immunity to helminths and drive allergic immune responses(4). ILC2s develop in the bone marrow via common ILC progenitor (CILP)(5), common helper ILC progenitor (CHILP)(6), innate lymphoid cell progenitor (ILCP) (7) and finally ILC2 progenitor (ILC2P)(8). Gata3 is critical to the development of ILCs (8, 9) and high levels are required for the development of ILC2P in the bone marrow (8, 10).

Dosage of Gata3 plays a critical role in T, NK and ILC development and peripheral immunity (8, 10, 11). To support this *Gata3* expression is spatiotemporally tuned by several distal enhancers that dictate *Gata3* activity in these individual lineages. Previous research identified a 7.1kb region ~280kb from the *Gata3* gene that is important for its expression in NK and T cell development, known as T cell enhancer 1 (Tce1**)** (12, 13). Subsequent work found that this region (also termed *Gata3* +278/285) also impacts ILC2 development but not Th2 cell differentiation (14). ILC2-specific effects have been attributed to additional distal regulatory regions, such as *Gata3* +674/762 (14) and the Gata3 tandem super enhancers (+500/764)(15). In addition, a Th2 specific enhancer (*Gata3* +906/935) has recently been identified (16). Overall, this complex regulatory landscape mechanistically fit with a dosage-threshold model controlling *Gata3* expression.

Recently we discovered a long non-coding RNA gene that we named *Distal regulatory enhancer of Gata3 1 (Dreg1)* which lies ~0.6kb upstream of Tce1 enhancer (17, 18). Expression of *Dreg1* is high in early T cell progenitors and Th2 cells, mirroring *Gata3* expression (17). Overexpression studies indicated that it may be involved in the establishment but not maintenance of *Gata3* expression (17, 18), but it remains unclear how the loss of *Dreg1* affects immune system development. Here, we explore the role of *Dreg1* in the regulation of immune cell differentiation. We find that deletion of the *Dreg1* locus leads to a specific loss of ILC2 progenitors and their downstream progeny in non-lymphoid tissues but not T or NK cells. Chromatin profiling reveals that the regulatory regions around the *Dreg1* gene are open in haematopoietic progenitors and are bound by the transcription factor TCF1 in early innate lymphoid progenitors and TCF1-deficient mice lack *Dreg1* expression. Finally, we discovered potential homologues of *Dreg1* that highly expressed in human ILC2 emanating from a syntenic enhancer of GATA3.

Results

Deletion of the Dreg1 locus results in a specific reduction of ILC2

To understand how *Dreg1* contributes to the development of the immune system we generated *Dreg1*-deficient mice by CRISPR-Cas9-mediated excision of the complete 3kb of the *Dreg1* gene (Figure 1A). We confirmed that the *Dreg1* gene was removed from the germline and found that these mice reproduced at Mendelian frequencies and exhibited no overt developmental defects suggesting that *Dreg1* is not required for embryogenesis.

Given our previous finding of the relatively high levels of *Dreg1* expression in T cells (17), and of a critical role of the Tce1 (*Gata3* +278/285) enhancer (12, 14), we expected that the deletion of *Dreg1* would impact T cell and NK cell development. We therefore performed an analysis of the different populations of T cells. Mature $CD4^+$ and $CD8^+$ T cells in the spleen appeared unaltered in frequency (Supplementary Figure 1). Moreover, both populations had similar proportions of naïve, effector, and memory cells (Supplementary Figure 1B &C). This suggests that *Dreg1* is not required for conventional T cell development or maintenance. In addition, the loss of *Dreg1* did not affect the development of NK cells and γδ T cells as normal proportions were observed in the spleen (Supplementary Figure 1D). Overall, this analysis suggests that *Dreg1* is dispensable for NK and T cell development.

Gata3 is also critical for the development of all innate lymphoid cells (ILCs)(9) and the levels of Gata3 are critical in directing ILC2 lineage development (8, 19). Therefore, we next explored peripheral sites known to harbour substantial numbers of these cell types such as visceral adipose tissue (VAT), the small intestine lamina propria (SI LP) and the lung. While these tissues had relatively normal proportions of NK, ILC1 and ILC3 (Figure 1B, C & E) we noted a specific reduction in the population of ILC2 in each of the tissues we examined (Figure 1D). Thus far the data obtained is from mice in which Dreg1 is deleted in all cells, hampering the ability to

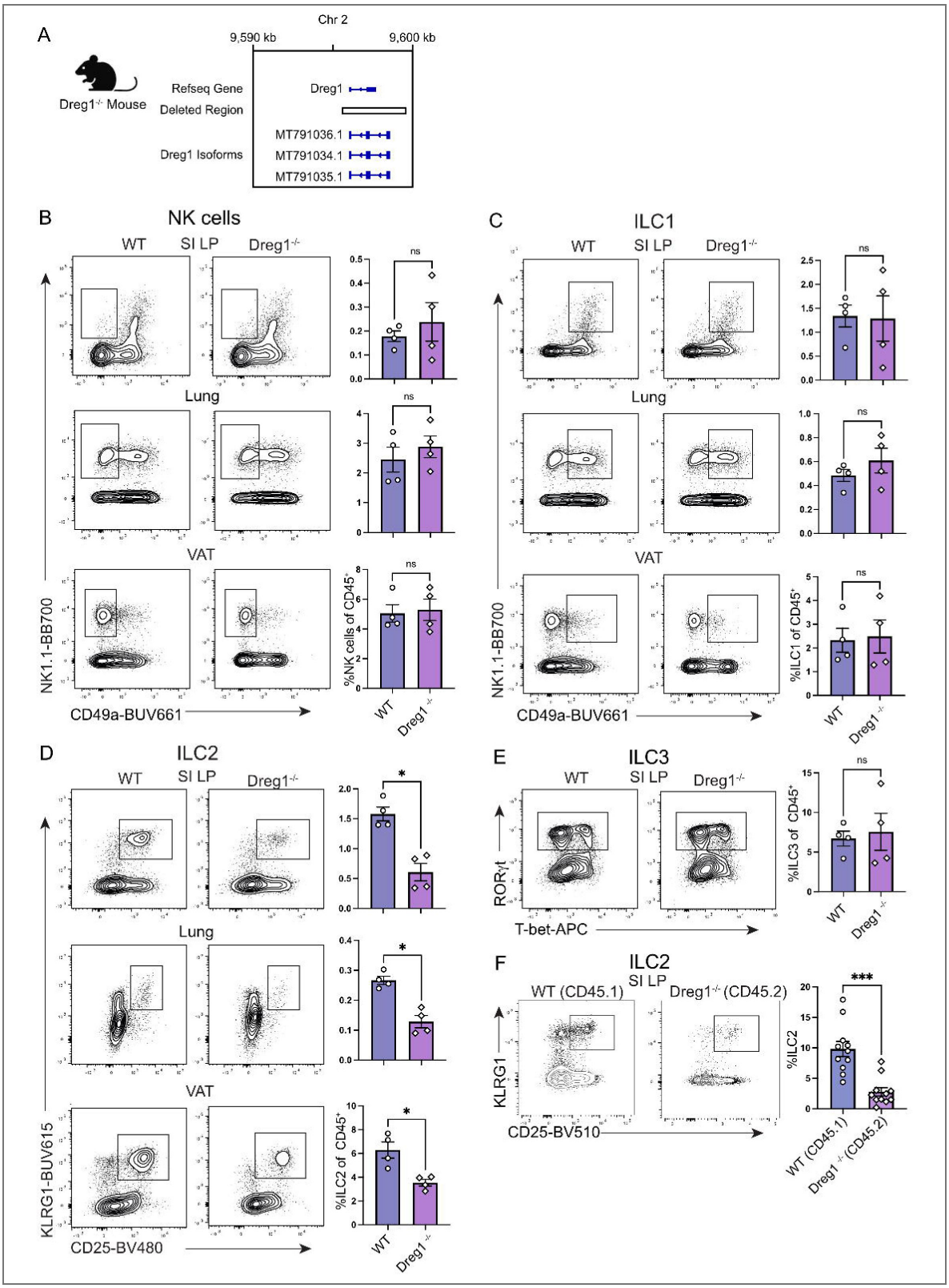


Figure 1. *Dreg1* deletion results in a specific reduction in peripheral ILC2 cells.

(A) Deletion of Dreg1 in mice using CRISPR-Cas9. (B-E) Representative FACS plots and % of CD45$^+$, TCRβ$^-$, CD19$^-$, CD11c$^-$ NK and ILC populations from the visceral adipose tissue (VAT), small intestinal lamina propria (SI LP), and lung from wild type (WT) or Dreg1-/- mice. Shown is one representative experiment of two with n=4 mice/group. (F) SI LP from mixed bone marrow chimeras were examined for the proportion of ILC2 from the wildtype (CD45.1) or Dreg1-deficient (CD45.2) compartment. Data is from pooled from 3 independent experiments. Mean and SEM together with individual data points are shown. Data were statistically analysed by Student *t* test.

determine whether the loss of Dreg1 intrinsically regulates ILC2 development or the phenotype is driven by extrinsic signals. To demonstrate a cell-intrinsic role of Dreg1 in ILC2s we generated mixed bone marrow irradiation chimeras comprising a ratio of 50:50 of wild type ($CD45.1^+$) to Dreg1-deficient ($CD45.2^+$) cells. In these mice we observed a reduction in Dreg1-deficient ILC2 in comparison to their wild type ILC2 counterparts in all tissues examined (Figure 1F and Supplementary Figure 1E-G). In contrast we observed comparable proportions of wild type and Dreg1-deficient B cells from these same tissues (Supplementary Figure 1F-G). Taken together these results show that the loss of *Dreg1* results in the specific and intrinsic loss of ILC2 in different peripheral tissues.

Dreg1 deficiency results in the reduction of ILC2 progenitors

We next investigated the origins of the ILC2 defect in the *Dreg1*-deficient mice. It is known that expression of *Gata3* in the early ILC progenitor stage is required for development of mature ILC2s (8, 10, 19). Indeed, analysis of publicly available RNAseq data (20) demonstrated that Gata3 expression was very high in ILC2P and ILC2 compared to other populations, whereas the highest expression of Dreg1 was found in ILC2P (Figure 2A). We examined ILC development in the bone marrow (Figure 2B-G, gating strategy in Supplementary Figure 2A). We began by examining the common lymphoid (CLP) and lymph-myeloid primed progenitors (LMPP) and found no overt changes in proportions or total number of these populations (Figure 2B & C). However, we found increased CILP, CHILP and ILCP in the *Dreg1*-deficient mice (reaching significance in the CILPs) (Figure 2D-F). We observed a substantial reduction of the ILC2P population suggesting that the loss of *Dreg1* leads to a block in ILC development (Figure 2G). This suggests that the loss of *Dreg1* results in a bottleneck at the early stages of ILC development. We next examined the levels of Gata3 in these populations (Figure 2H). We observed a reduction in Gata3 signal in the upstream CHILP and the ILCP from the *Dreg1*-deficient mice, but interestingly Gata3 levels were normal in the ILC2P even though this population is reduced in the *Dreg1*-deficient mice. This suggests that *Dreg1* acts to regulate Gata3 prior to the ILC2P stage which leads to a block in their development, however, if cells can overcome this perturbation, they express relatively normal levels of Gata3.

Dynamic epigenetic regulation of the Dreg1 locus in a Tcf1-dependent manner during ILC development

To gain a greater understanding of how the expression of *Dreg1* is regulated we next studied the chromatin state of the *Dreg1* locus during early ILC development via analysis of publicly available ATAC-Seq data generated on progenitors and their downstream ILC progeny (14)(Figure 3A). Of note, the original Refseq annotation of Dreg1 was missing the first exon and TSS which we identified (17) and show in Figure 3A. We observed three significant accessible chromatin "peaks", with the predominant one at the promoter which was open at the CLP stage remained so in ILCP and ILC2P. One region downstream became more accessible in ILCP and ILC2P in line with that observed by others (14). Overall, we find the promoter of *Dreg1* is open in early lymphoid progenitors and the acquisition of chromatin accessibility downstream correlates with increased *Dreg1* expression in ILC2 progenitors.

To understand the transcription factors that might be involved in regulating *Dreg1* expression we examined the motifs at the *Dreg1* promoter and downstream accessible region. We observed a specific enrichment of motifs for Tcf1, Lef1 and Ets1 suggesting that these transcription factors may be involved in regulating *Dreg1* expression. As Tcf1 (encoded by the *Tcf7* gene) has been implicated in ILC2 development (21, 22) we focussed on its role in regulating *Dreg1* expression. Firstly, we examined publicly available Tcf1 CUT&Run data (23) and found that indeed Tcf1 was specifically bound to the accessible sites of the *Dreg1* locus in early innate lymphoid progenitors (EILP, which are a population that sit between CILP and CHILP) (Figure 3B). We next examined ChIC-Seq data (24) of active (H3K27ac) or repressive (H3K27me3) histone modifications at different stages of ILC development (Figure 3D). Firstly, we found no evidence of H3K27me3 in this region in any of the cell types examined. However, there was a modest accumulation of H3K27ac around

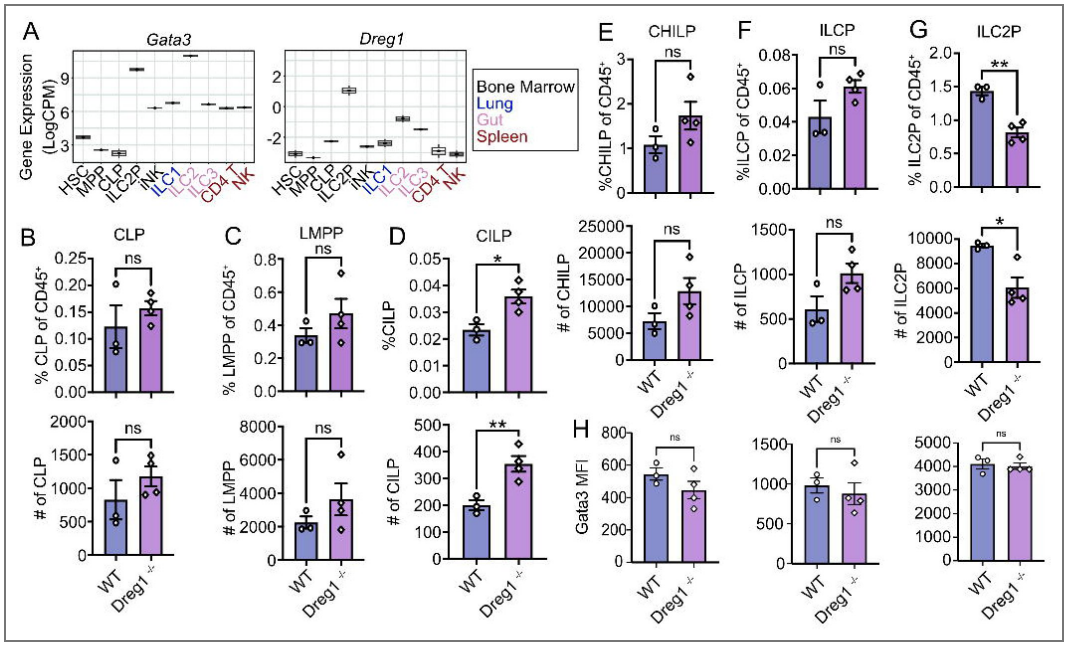


Figure 2. *Dreg1* deletion results in a bottleneck in ILC development and a reduction in ILC2P in the bone marrow.

(A) Boxplots showing expression of *Gata3* and *Dreg1* from RNA-Seq (GSE77695). (B-G) Quantification of ILC progenitor subsets in the bone marrow with percentages (upper) and total numbers (below). (H) Mean fluorescence intensity (MFI) of Gata3 measured on CHILP, ILCP and ILC2P. Shown is one representative experiment of two with n=3-4 mice/group. Mean and SEM together with individual data points are shown. Data were statistically analysed by Student *t* test.

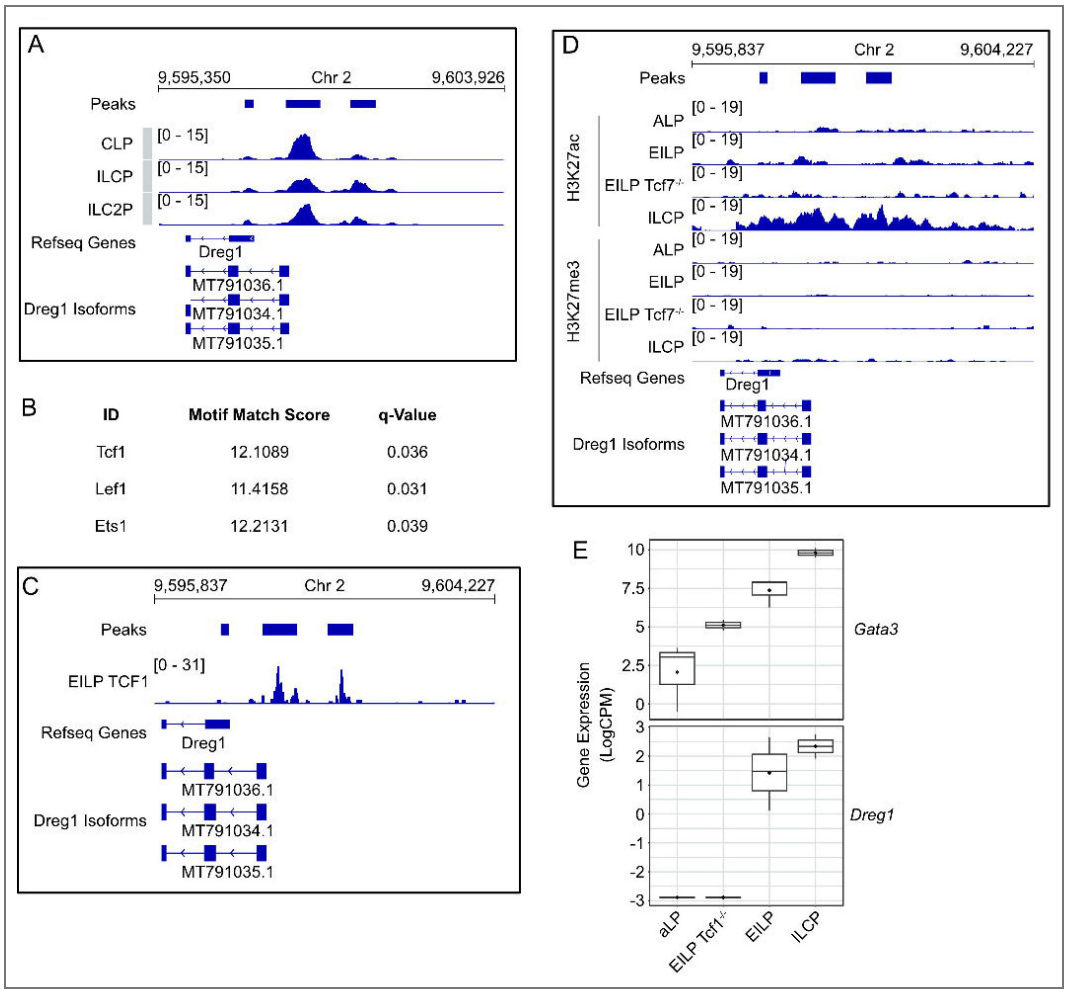


Figure 3. *Dreg1* locus is dynamically regulated in a Tcf1-dependent manner during ILC2 development.

(A) Chromatin accessibility around Dreg1 in ILC progenitors (GSE169542). (B) Motifs enriched in accessible regions around Dreg1. (C) Tcf1 binding around Dreg1 locus in EILP (GSE128483). (D) Histone marks around Dreg1 at different stages of ILC development and in Tcf7-/- EILP (GSE142468). (E) *Gata3* and *Dreg1* expression quantified from RNA-Seq data in ILC progenitors and Tcf7-/- EILP (GSE113767).

the accessible regions in the *Dreg1* locus at the EILPs which was noticeably increased at the ILCP stage. Of note, Tcf1-deficient (via deletion of the *Tcf7* gene) EILP showed lower levels of H3K27ac suggesting that Tcf1 is likely involved in creating a permissive chromatin landscape at the *Dreg1* locus during ILC development. Indeed, the expression of *Dreg1* was dependent on the presence of Tcf1 as *Tcf7*-/- EILPs lacked *Dreg1* expression (23)(Figure 3E). Taken together this data suggests that the alterations to the chromatin state *Dreg1* locus and its subsequent transcriptional activity during ILC development is dependent on Tcf1.

Identification of a human GATA3 enhancer harbouring Dreg1 homologues that are active in ILC2s

Previously we identified a region syntenic to the mouse *Dreg1* locus on human chromosome 10 that is a similar distance from the *GATA3* gene and forms an 3D interaction in T cells but not B cells suggesting it may a represent an enhancer element (17). Interestingly, bidirectional transcription initiates from the sequence-conserved *cis* element to produce two potential human homologs of *Dreg1*, the lncRNAs CAT00000105356.1 (we named *DREG1.1*) and CAT00000117261.1 (*DREG1.2*). To explore whether these transcripts are also expressed in ILC populations we examined publicly available RNAseq data from healthy human blood (25)(Figure 4A). We found that GATA3 was highly expressed in human ILCP, ILC2 and TH2 cells. We also observed this pattern for *DREG1.1* and to an even greater extent for *DREG1.2* suggesting that the syntenic region harbours transcripts which reflect the same expression pattern as murine *Dreg1*.

Finally, we investigated the functional role of this potential enhancer of GATA3 by examining a recently published tiling CRISPR deletion screen searching for regulatory elements that control GATA3 expression in human Th2 cells (26). We focused on the syntenic region which was defined as functional sequence (FS) 23 (Figure 4B). Specifically, guides that deleted conserved sequences FS23-4 and FS23-5 led to a significant downregulation of GATA3 expression (26) suggesting that this region represents a distal enhancer of GATA3 (Figure 4B). ATACseq data from either cultured (pcILC2) or fresh ILC2 from healthy human blood (27) revealed a clear accessible region in these cells that overlaps the *DREG1.1/2* and FS23 (Figure 4C). Overall, we find that this syntenic region in humans represents an enhancer that is important for high levels of *GATA3* expression and contains two non-coding RNA genes that show a similar expression pattern to murine *Dreg1*.

Discussion

Here we show that germline excision of the *Dreg1* locus resulted in a specific reduction in ILC2 cells owing to a developmental bottleneck during ILC development in the bone marrow. These results are in line with the fact that very high levels of Gata3 are required for ILC2 development (8, 10). Given its proximity to the Tce1 (+280kb *Gata3*) enhancer, a surprising finding was that the deletion of *Dreg1* did not affect T or NK cells lineages. While it is tempting to speculate that the *Dreg1* locus represents a ILC2-specific enhancer element we favour the idea that *Dreg1* likely contributes to dosage tuning of Gata3 during ILC2 commitment and that ILC2s because of their higher Gata3 threshold are more sensitive to modest reductions, whereas T and NK cells are buffered by redundant modules.

Our analysis of publicly available data revealed that the Dreg1 locus was accessible in common lymphoid progenitors and subsequently gained H3K27ac during ILC differentiation coinciding with increased Dreg1 expression. This region was enriched for Tcf1 motifs which was supported by analysis of data from Harly et al.(23), that showed indeed that Tcf1 bound to these regions in ILCP. Subsequent analysis revealed that H3K27ac of the Dreg1 locus and the expression of Dreg1 was reduced in Tcf1-/- EILP. Taken together this suggests that Tcf1, which is known to be critical for Gata3 expression during ILC development is upstream of, and may act in concert with Dreg1 to promote high levels of Gata3 expression in ILC2 development.

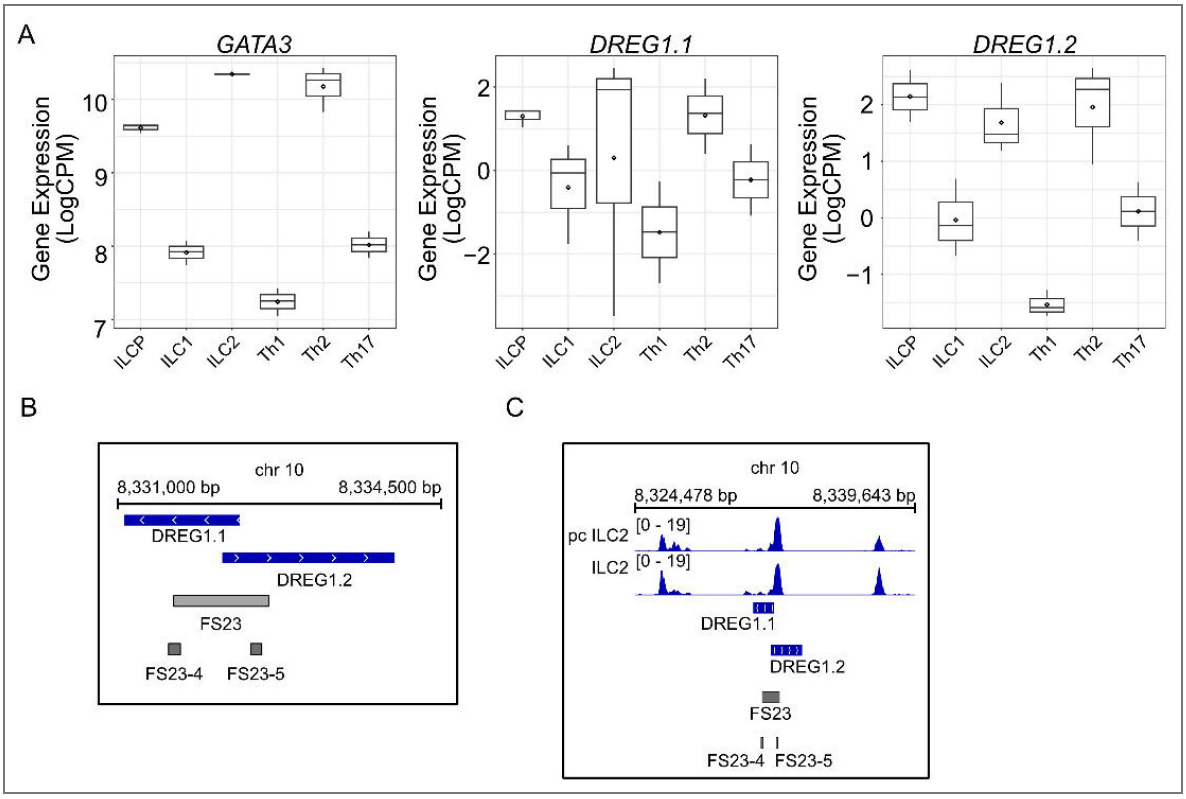


Figure 4. A syntenic region in humans represents a *GATA3* enhancer element with a transcriptional profile akin to murine *Dreg1*.

(A) *GATA3* and *DREG1.1 and DREG1.2* expression quantified from RNA-Seq data in ILC and T helper populations from isolated from healthy human blood (PRJEB35186). (B) Alignment of Functional Sequence 23 (FS23) that overlaps the syntenic region revealed as regulating GATA3 expression in a CRISPR deletion screen of human TH2 cells (26). (C) Chromatin accessibility around FS23 guides from the CRISPR deletion screen (26) (GSE231999).

While the analysis of publicly available data was invaluable in providing insights into the regulation of Dreg1, further experiments will dig deeper into the mechanisms utilised by Dreg1 to control Gata3 expression. Indeed, how *Dreg1* is involved in regulating *Gata3* levels mechanistically is yet to be determined. *Dreg1* may act as an enhancer RNA that cooperates with chromatin modifying proteins (28) to reinforce local H3K27ac and enhancer-promoter communication. Future experiments that selectively suppress *Dreg1* transcription for example via antisense oligonucleotides or CRISPRi at the Dreg1 promoter will enable the discrimination of RNA-dependent from DNA element-dependent effects.

In humans, we identify two noncoding transcripts within the syntenic GATA3 distal enhancer that are highly expressed in ILCP, ILC2 and TH2 cells. Perturbations within this conserved block in an independent tiling-deletion screen performed by Chen et al.(26), resulted in reduced GATA3 expression in T cells. While these experiments were performed in T cells, we expect that this enhancer region will likely also play an important role in the regulation of ILC2 development. Given that ILC2 have been implicated in the development of allergic reactions (4) the noncoding transcripts we identified may represent targets for the modulation of GATA3 levels and ILC2 function in disease.

Methods

Mice

The *Dreg1* knockout mouse line was generated at the Melbourne Advanced Genome Editing Centre (MAGEC) laboratory using CRISPR-Cas9 through microinjection into the pronucleus of one-cell stage C57BL/6 embryos with 2 sgRNAs (CTACTTGCTGACAAGTCGTC and TCTAGTAAGTCCAGTTGCTT) to target the 3,594 bp genomic region around *Dreg1*. The F0 offspring were validated for *Dreg1* deletion with forward primer GACCAGATATGGAGACGTGCA and reverse primer TCTTTGCCATCTTC TGTGTGC and were backcrossed with wild-type C57BL6/J mice for 2 or more generations.

Cells and tissues were obtained from littermate control mice aged at least 6 weeks unless otherwise specified. Due to sex differences in ILC populations (29), only female mice were used. All mice were maintained at the WEHI Animal Facility under specific pathogen-free conditions.

Bone marrow chimeras

Mixed bone marrow chimeras were generated by harvesting bone marrow from either wild type ($CD45.1^+$) mice or Dreg1-deficient ($CD45.2^+$) mice, mixing equally and injecting 5 million cells intravenously into lethally irradiated (2x550 Gy) F1 ($CD45.1^+/CD45.2^+$) mice. Mice were left to reconstitute for over 8 weeks.

Cell isolation

Thymus and spleen

The spleen and thymus were made into single-cell suspension by mashing through a 70μm mesh. Splenic cells were treated with red cell lysis buffer.

Bone marrow

Both pairs of hips, femurs, and tibia bones were grounded in a mortar and pestle to collect the bone marrow. The cell suspension was filtered through a 70μm cell strainer and negatively enriched for ILC cells with the mouse lineage cell depletion kit (Miltenyi).

Extraction of lymphocytes from non-lymphoid tissues

Lymphocytes from the VAT and lung were finely chopped in 3-5mL digestion buffer (RPMI and 2% FCS with 2mg/mL Collagenase IV). The suspension was digested for 40 min at 37°C and shaken at 180 rpm. Digested VAT samples were topped up to 30ML with FACS buffer (1xPBS with 2% FCS) and spun at 800g for 15 min at 4°C. Lungs were mashed through a 70mM filter and topped to 30mL FACS buffer and pelleted at 1700rpm for 5min. Both VAT and lung pellets were treated with RBC lysis buffer.

Isolation of lymphocytes from small intestinal lamina propria

Mesentery and Peyer's patches were removed longitudinally, and faeces removed. Intestine was chopped into 0.5cm pieces into 1xPBS and vortexed. Intestinal pieces were placed into 20mLs of dissociation buffer (1xHBSS without Ca2+ and Mg2+ with 2% FCS, 10mM HEPES and 5 mM EDTA) and digested for 40min at 37°C at 180rpm. Digested pieces were washed with FACS buffer and then placed in 5-8mL of digestion buffer (1xHBSS with indicator containing 2% FCS, 10mM HEPES, 2mg/mL Collagenase IV and 2mg/mL DNAse I) and incubated for 30-40min at 37°C at 180rpm. Digested tissue was mashed through a 70 mM cell strainer and pelleted. A 40/80% percoll gradient was performed to enrich for lymphocytes.

Flow cytometry and antibodies

Fluorophore conjugated antibodies against mouse antigens were used for flow cytometry. Antibodies, clone names and manufactures: ***Biolegend***- CD4 (GK1.5) APCFire750, CD8a (53-6.7) BV570, NKp46 (29A1.4) PEcy7, ICOS (7E.17G9) BV421, Ki67 (11F6) BV650, CD62L (MEL-14) APC and T-bet (4B10) APC. ***BD***- CD19 (1D3) BV786, TCRb (H57-597) BV750, TCRgd (GL3) BV711, B220 (RA3-6B2) BV786, GATA3 (L50-823) BUV395, RORgt (Q31-378) BV421, RORgt (Q31-378) PerCPcy5.5, KLRG1 (2F1) BUV615, CD49a (Ha31/8), CD25 (PC61) BV480, NK1.1 (PK136) BUV805, NK1.1 (PK136) BB700, CD44 (IM7) BUV496. **Invitrogen/bioscience**- CD11c (N418) FITC, FOXP3 (FJK-16s) PE, IL-33R (ST2) (RMST2-2) PerCP-eFLuor710, Streptavidin BUV563.***WEHI***-CD45.2 (104) AF700. Live dead staining was performed using Fixable viability stain 440UV diluted in 1xPB at 1:1000 dilution. Surface staining of antibodies was for 30min on ice at 1/200, 1/300 or 1/400 in FACS buffer. Intracellular staining of transcription factors was overnight at 4°C using the Foxp3/Transcription factor staining buffer set from eBioscience overnight. Cell numbers for specific cell populations were calculated using the volume of the sample that was analyzed, the cell counts per sample, and the percentage of the specific cell population.

RNA-Seq Analysis

Publicly available RNA-Seq data were downloaded from SRA and re-analysed. Briefly fastq files were analyzed with FASTQC (0.12.1) and adapters were removed using trimmomatic(30) (0.36). The reads were aligned to GRCm39 for mouse samples and GRCh38 for human samples using hisat2(31) (2.0.5). Gene counts were quantified using Rsubread(32) featureCounts (1.6.3) with annotation from gencode(33) (vM33 for mouse and v42 for human), which had the annotation for the *Dreg1* gene or human homologs of the *Dreg1* gene added, respectively. Samples were processed using edgeR(34) (3.42.4). Lowly expressed genes were removed, and samples were normalized according to library size. Gene expression was plotted as logCPM using ggplot2 (3.4.4).

ATAC-Seq Analysis

Publicly available ATAC-Seq data were downloaded from SRA and re-analysed. Processing was based on the ENCODE ATAC-Seq pipeline. Briefly fastq files were analyzed with FASTQC and adapters were removed using trimmomatic (0.36). The reads were aligned to the GRCm39 gnome for mouse samples and GRCh38 genome for human samples using bowtie2 (2.4.4). Duplicate reads were marked using Picard tools (2.26.11) and reads were filtered using samtools(35) (1.9) with -F 1804 -f 2. Fold-change coverage tracks were created using macs2 (2.2.7.1) callpeak with a smooth window of 150 and a shift size of -75 and bdgcmp. The bedgraph was converted to a bigwig using ucsc tools (331) bedGraphToBigWig(36) and visualized using IGV(37) (2.17.4) with group scaling among samples from the same experiment.

Motif Scanning

The regions of interest as identified through peak calling of ATAC-Seq data were scanned for motifs using FIMO(38) from the MEME suite (5.0.5) using the Hocomoco(39) (H12) core motif database. The resulting list was filtered to remove factors that were not expressed in ILC2P cells as determined through bulk RNA-Seq (GSE77695) with a cutoff of >= 1 LogCPM.

ChIC and CUT&Run Analysis

Publicly available ChIP-Seq or CUT&Run data were downloaded from SRA and re-analysed. Briefly fastq files were analyzed with FASTQC and adapters were removed using trimmomatic (0.36). The reads were aligned to the GRCm39 gnome for mouse samples using bowtie2 (2.4.4) with the options -q -5 0 -3 0. Duplicate reads were marked using Picard tools (2.26.11) and reads were filtered using samtools (1.9) with -F 1804 -f 2. Fold-change coverage tracks were created using macs2 (2.2.7.1) callpeak with a smooth window of 50 and a shift size of -25 for transcription factors and 150 and -75 for histone marks along with macs2 bdgcmp. The bedgraph was converted to a bigwig using ucsc tools (331) bedGraphToBigWig and visualized using IGV (2.17.4) with group scaling among samples from the same experiment.

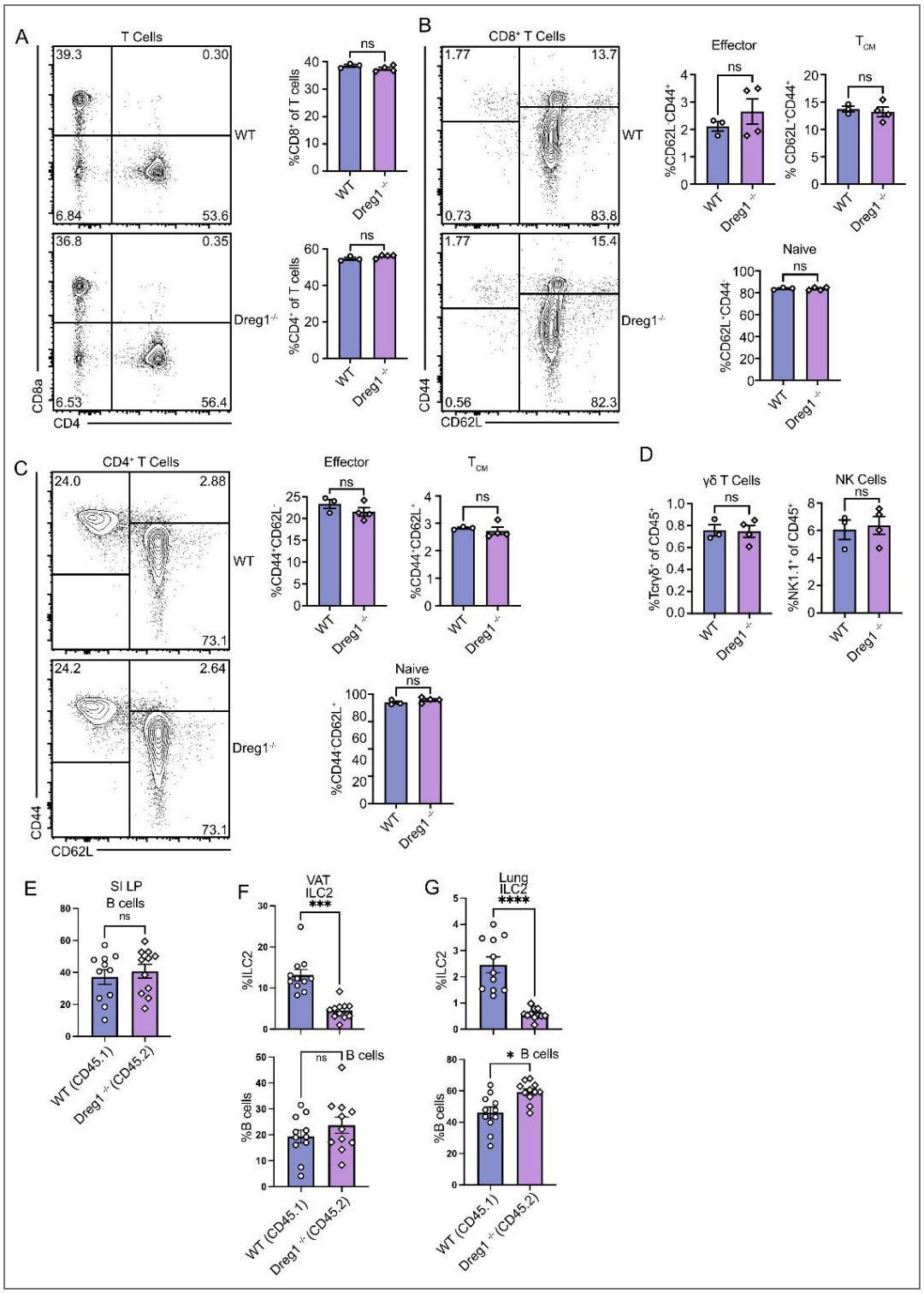
CRISPR Guides

The guide counts were downloaded from GSE190860 and were used to create a bed file to identify the target regions. The bed file was visualized in IGV (2.17.4).

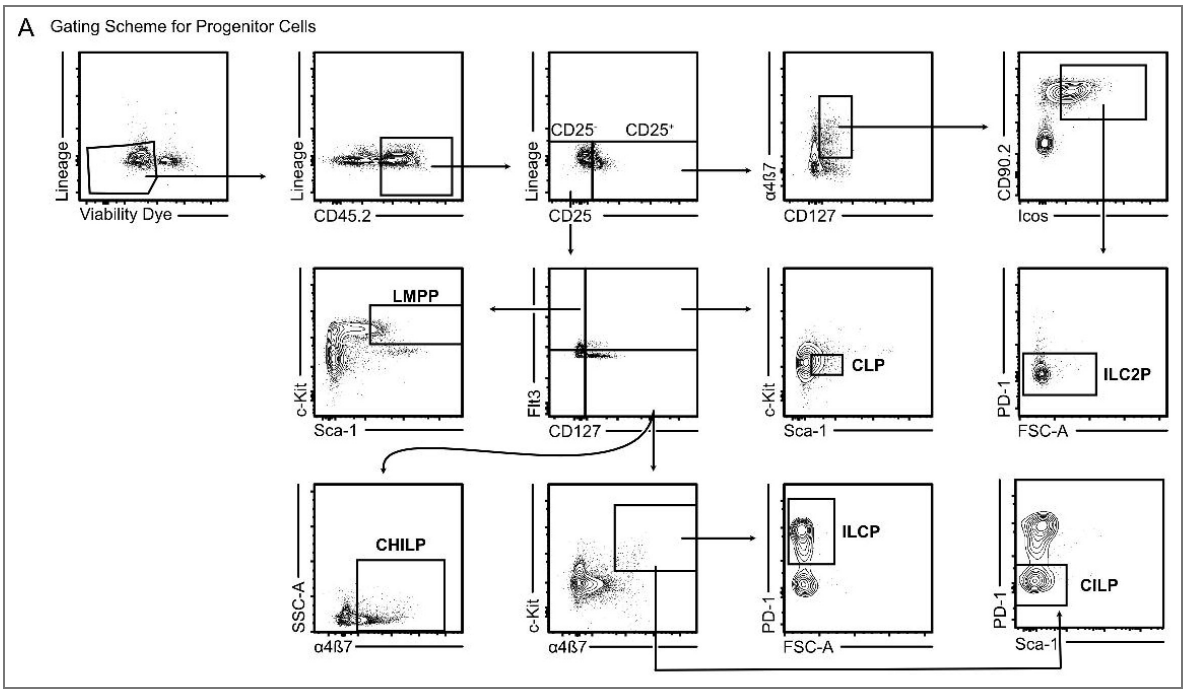
Publicly available datasets used in this study

Dataset type	Accession Number
Bulk RNA-Seq (Mouse-ILC) (20)	GSE77695
Bulk RNA-Seq (Mouse-ILC w TCF1KO) (23)	GSE113767
Bulk RNA-Seq (Human) (25)	PRJEB35186
Bulk ATAC-Seq (ILCP) (14)	GSE169542
Bulk ATAC-Seq (HumanMouseILC2) (27)	GSE231999
Bulk ChIP-Seq (Histone) (40)	GSE85156
Bulk CUT&Run (EILP TCF1) (23)	GSE128483
Bulk ChIC-Seq (Histone Tcf1 KO) (24)	GSE142468
CRISPR Screen (26)	GSE190860

Supplementary figures



Supplemental Figure 1. ***Dreg1*** **deletion in does not affect T or NK cells.** (A-C) Representative FACS plots with quantification (right) showing expression of (A) CD8α and CD4 in splenic T cells. (B) CD44 and CD62L in splenic $CD8^+$ T cells (C) CD44 and CD62L in splenic $CD4^+$ T cells. (D) Quantification of splenic γδ T cells and NK cells. Shown is one representative experiment of two with n=3 mice/group. Mixed bone marrow chimeras were examined for the proportion of SI LP B cells (E), VAT ILC2 or B cells (F) or Lung ILC2 or B cells from the wildtype (CD45.1) or Dreg1-deficient (CD45.2) compartment. Data is from pooled from 3 independent experiments. Mean and SEM together with individual data points are shown. Data were statistically analysed by Student *t* test.



Supplemental Figure 2. Gating of ILC progenitors in bone marrow.

(A) Flow cytometric analysis of bone marrow cells was used to quantify the changes between wild type and Dreg1-deficient haematopoietic and ILC progenitors in Figure 2. Viable, lineage negative, CD45.2+ bone marrow cells were gated by the expression of cell surface markers into the delineated progenitor populations, and this was used to quantify their proportions and numbers.

Data availability

All data generated or analyzed during this study are included in the manuscript and supporting files; source data files have been provided for all figures.

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Authorship contributions

Sara Quon: Conceptualization, Investigation, Formal Analysis, Writing-Original Draft, Review & Editing. **Adelynn Tang:** Conceptualization, Investigation, Formal Analysis, Writing-Review & Editing. **Nadia Iannarella**: Investigation. **Kael Schoffer:** Investigation. **Wing Fuk Chan:** Conceptualization, Methodology. **Timothy Johanson:** Investigation, Supervision. **Ajith Vasanthakumar:** Conceptualization, Supervision, Funding Acquisition, Writing-Review & Editing. **Rhys Allan:** Conceptualization, Supervision, Funding Acquisition, Writing-Original Draft, Review & Editing.

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Note

This reviewed preprint has been updated to correct author email address.

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Peer reviews

Reviewer #1 (Public review):

Summary:

This study examines the role of the long non-coding RNA Dreg1 in regulating Gata3 expression and ILC2 development. Using Dreg1 deficient mice, the authors show a selective loss of ILC2s but not T or NK cells, suggesting a lineage-specific requirement for Dreg1. By integrating public chromatin and TF-binding datasets, they propose a Tcf1-Dreg1-Gata3 regulatory axis. The topic is relevant for understanding epigenetic regulation of ILC differentiation.

Strengths:

(1) Clear in vivo evidence for a lineage-specific role of Dreg1.

(2) Comprehensive integration of genomic datasets.

(3) Cross-species comparison linking mouse and human regulatory regions.

Weaknesses:

(1) Mechanistic conclusions remain correlative, relying on public data.

(2) Lack of direct chromatin or transcriptional validation of Tcf1-mediated regulation.

(3) Human enhancer function is not experimentally confirmed.

(4) Insufficient methodological detail and limited mechanistic discussion.

Comments on revisions:

The authors have provided clear evidence that Dreg1 is necessary for ILC2 development, but their refusal to perform any mechanistic experiment remains a significant weakness. While their appeal to the 3Rs and the use of public datasets is noted, re-analyzing external data from heterogeneous sources cannot substitute for direct, internal validation of the Tcf1-Dreg1-Gata3 axis in their specific knockout model. This is particularly problematic because ILC2 progenitors, though rare, can be isolated from bone marrow, especially since assays like CUT&Tag and others are specifically designed for low cell numbers. By relying on public T-cell CRISPR screens to justify human ILC2 functions, the authors are substituting cross-cell-type correlation for definitive functional proof. Consequently, the manuscript currently describes a discovery of necessity without providing a verified molecular mechanism, which should be more explicitly reflected in the title and conclusions.

https://doi.org/10.7554/eLife.109408.2.sa2

Reviewer #2 (Public review):

The authors investigate the role of the long non-coding RNA Dreg1 for the development, differentiation or maintenance of group 2 ILC (ILC2). Dreg1 is encoded close to the Gata3 locus, a transcription factor implicated in the differentiation of T cells and ILC, and in particular of type 2 immune cells (i.e., Th2 cells and ILC2). The center of the paper is the generation of a Dreg1-deficient mouse. The role of Dreg1 in ILC2 was documented by mixed bone marrow experiments. While Dreg1-/- mice did not show any profound ab T or gd T cell, ILC1, ILC3 and NK cell phenotypes, ILC2 frequencies were reduced in various organs tested (small intestine, lung, visceral adipose tissue). In the bone marrow, immature ILC2 or ILC2 progenitors were reduced whereas a common ILC progenitor was overrepresented suggesting a differentiation block. Using ATAC-seq, the authors find the promoter of Dreg1 is open in early lymphoid progenitors and the acquisition of chromatin accessibility downstream correlates with increased Dreg1 expression in ILC2 progenitors. Examining publicly available Tcf1 CUT&Run data, they find that Tcf1 was specifically bound to the accessible sites of the Dreg1 locus in early innate lymphoid progenitors. Finally, the syntenic region in the human genome contains two non-coding RNA genes with an expression pattern resembling mouse Dreg1.

The topic of the manuscript is interesting. The article is focused on the first description of the Dreg1 knockout mouse and the specific effect of Dreg1 deficiency on ILC2 development.

(1) The data of how Dreg1 contributes to the differentiation and or maintenance of ILC2 is not addressed at a very definitive level. Does Dreg1 affect Gata3 expression, mRNA stability or turnover in ILC2? Previous work of the authors indicated that knock-down of Dreg1 does not affect Gata3 expression (PMID: 32970351). The current data (Figure 2H) showed small differences in Gata3 expression in CHILP which were, however, not statistically significant. No differences were found in ILCP and ILC2P.

(2) How Dreg1 exactly affects ILC2 differentiation remains unclear.

https://doi.org/10.7554/eLife.109408.2.sa1

Author response:

The following is the authors' response to the original reviews

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *This study examines the role of the long non-coding RNA Dreg1 in regulating Gata3 expression and ILC2 development. Using Dreg1-deficient mice, the authors show a selective loss of ILC2s but not T or NK cells, suggesting a lineage-specific requirement for Dreg1. By integrating public chromatin and TF-binding datasets, they propose a Tcf1-Dreg1-Gata3 regulatory axis. The topic is relevant for understanding epigenetic regulation of ILC differentiation.*
>
> *Strengths:*
>
> *(1) Clear in vivo evidence for a lineage-specific role of Dreg1.*
>
> *(2) Comprehensive integration of genomic datasets.*
>
> *(3) Cross-species comparison linking mouse and human regulatory regions.*
>
> *Weaknesses:*
>
> *(1) Mechanistic conclusions remain correlative, relying on public data.*

We agree that the mechanistic conclusions are of our study are indeed correlative and we mention this in the discussion. The primary work of the study is the discovery of *Dreg1*'s necessity for ILC2 development via the new knockout mouse model. Re-analysing good quality publicly available data on rare cell populations is an appropriate approach and in line with DORA guidelines for ethical research.

> *(2) Lack of direct chromatin or transcriptional validation of Tcf1-mediated regulation.*

The most appropriate way to examine direct Tcf1 target genes in primary cells is to examine the association of Tcf1 binding with the changes that occur in Tcf1-bound genes after *Tcf7* knockout. By analysing publicly available data on ILC progenitors we indeed did this. We revealed that Tcf1 bound to *Dreg1* and that *Dreg1* was not expressed when Tcf1 was knocked out in ILC progenitors. In addition we examined H3K27ac at the *Dreg1* locus in the same ILC progenitors to demonstrate that Tcf1 appears to be important for decorating the *Dreg1* gene with this histone modification. We believe that this analysis is sufficient to conclude that Tcf1 is required for the expression of *Dreg1* in ILC progenitors.

> *(3) Human enhancer function is not experimentally confirmed.*

We agree that the potential human enhancer of GATA3 we identified has not been confirmed in human ILC. However, a previous study showed clear evidence that this region has GATA3 enhancer activity in human T cells. Therefore, while not specific to ILC2s the region where the DREG1 homologues lie does indeed harbour enhancer activity.

> *(4) Insufficient methodological detail and limited mechanistic discussion.*

We have now made the changes suggested by the reviewer to both the methods/figure legends and also the discussion.

> ***Reviewer #1 (Recommendations for the authors):***
>
> *The authors generated Dreg1-deficient mice and demonstrated that loss of this locus selectively reduces ILC2s but not T or NK cells, indicating a lineage-specific requirement for Dreg1 in ILC development. By analyzing publicly available chromatin accessibility and*

> *transcription factor-binding datasets, they link Dreg1 expression to Tcf1-dependent chromatin activation and extend their findings to human data by identifying a syntenic GATA3 enhancer that produces homologous Dreg lncRNAs in ILC2s. While the study addresses an interesting question, most of the mechanistic interpretations rely heavily on publicly available datasets rather than the authors' own functional evidence. To establish causality and reinforce the overall conclusions, I provide below some comments and suggestions for additional experiments and clarifications that would considerably strengthen the manuscript.*
>
> *(1) In Figure 3, the authors use public datasets to argue that Tcf1 regulates Dreg1 expression by modulating chromatin accessibility and H3K27ac at its locus. However, since these data are derived from heterogeneous external sources, the conclusions remain associative. To better support causality, the authors should generate matched datasets from their own sorted progenitor populations and perform CUT&Tag for Tcf1 and H3K27ac in wild-type and Tcf7 knockout progenitors to directly test whether Tcf1 binding establishes an active chromatin state at Dreg1. Also, complementing this with nascent RNA or pre-mRNA quantification would link chromatin activation to transcriptional output. These experiments are technically feasible in progenitors and would substantially strengthen the claim that Tcf1 directly drives Dreg1 activation during ILC development.*

We believe that utilising publicly available data sufficiently answers this question while also adhering to ethical considerations. The ILC populations used to produce the publicly available data were akin to those we examined in our analyses, and the data was of sufficient quality. Moreover, they enable us to access data from Tcf1-deficient mice. Redoing large-scale chromatin profiling on rare cell types would require hundreds of mice to achieve sufficient cell numbers. Repeating this solely for “originality” contradicts the 3Rs principles (replacement, reduction, refinement) if high quality public data already exists and we feel will require years of redundant work. In addition, we believe the fact that the data derive from heterogenous external sources, yet align well, only strengthen our conclusions. We have now added mention to our use of publicly available data in the discussion.

> *(2) In Figure 4, the authors provide correlative evidence from public datasets suggesting that the human region syntenic to the murine Dreg1 locus acts as a distal enhancer of GATA3 and gives rise to two ILC2-specific lncRNAs. To substantiate this claim, the authors should perform CUT&Tag for H3K27ac in human ILC2s to confirm enhancer activation and use 3C or HiChIP to demonstrate physical interaction with the GATA3 promoter. These experiments should be doable by fusing pooled ILC2 samples and would provide more direct evidence that this region actively regulates GATA3 expression.*

Assessing the activity of a distal enhancer region on its target gene in primary human cells is extremely difficult, due to a number of technical and biological complications such as enhancer redundancy. This is why we chose to reanalyse an extensive enhancer deletion screen performed in human T cells by Chen et al., AJHG 2023. This analysis clearly showed deletion of the region we identified as harbouring *Dreg1* homologues affected GATA3 expression, thus confirming its enhancer activity. While we agree with the reviewer that specific profiling of human ILC populations for H3K27ac and 3D genome architecture would provide further correlative evidence this will be a time-consuming and costly endevour with human material and ultimately the definitive proof in ILCs would require specific deletion of this region in ILC2s. We have mentioned this caveat in the discussion.

> *(3) Several figure legends lack essential methodological details. Figure 1 should specify how NK and ILC populations were gated, including intermediate steps and markers used. The same applies to Supplementary Figure 1, and particularly to Supplementary Figure 2, where gating strategies for progenitors are shown but not explained. Figure 2 should*

> *also indicate that these analyses were performed in bone marrow. Clearer legends are crucial for interpreting and reproducing the data.*

We have made the suggested changes.

> *(4) It is also unclear throughout the manuscript whether the authors performed any ATACseq experiments themselves or relied entirely on public datasets. This information should be stated explicitly in the main text and figure legends, not only in the Methods section. Similarly, the source of the ChIPseq or CUT&Run datasets should be clearly indicated alongside the relevant figures.*

We apologise for not making this clearer and have now clearly articulated if the data was public in the text.

> *(5) As the authors themselves suggest, performing experiments that selectively suppress Dreg1 transcription using antisense oligonucleotides or CRISPR interference at the Dreg1 promoter would provide more valuable mechanistic insights. Conducting these experiments in their own system would allow them to determine whether Dreg1 functions through its RNA product or as a DNA enhancer element, thereby strengthening the causal link between Dreg1 activity and Gata3 regulation.*

We agree with the reviewer, however, this, in our opinion is beyond the scope of this manuscript. The strength of this manuscript lies in the findings from the novel *Dreg1* knockout mouse strain. Future studies will focus on understanding how *Dreg1* influences Gata3 expression.

> *(6) The discussion would benefit from a clearer and more integrated explanation of how Dreg1 fits into the transcriptional network that controls ILC2 differentiation. The authors could elaborate on whether Dreg1 fine-tunes Gata3 expression or functions as part of a regulatory loop with Tcf1, and better explain how this mechanism might be conserved in humans. In addition, the authors should explicitly acknowledge the limitations of relying on publicly available datasets and emphasize the need for direct experimental validation to support their mechanistic interpretation.*

We have now made these suggested inclusions.

> ***Reviewer #2 (Public review):***
>
> *The authors investigate the role of the long non-coding RNA Dreg1 for the development, differentiation, or maintenance of group 2 ILC (ILC2). Dreg1 is encoded close to the Gata3 locus, a transcription factor implicated in the differentiation of T cells and ILC, and in particular of type 2 immune cells (i.e., Th2 cells and ILC2). The center of the paper is the generation of a Dreg1-deficient mouse. While Dreg1-/- mice did not show any profound ab T or gd T cell, ILC1, ILC3, and NK cell phenotypes, ILC2 frequencies were reduced in various organs tested (small intestine, lung, visceral adipose tissue). In the bone marrow, immature ILC2 or ILC2 progenitors were reduced, whereas a common ILC progenitor was overrepresented, suggesting a differentiation block. Using ATAC-seq, the authors find that the promoter of Dreg1 is open in early lymphoid progenitors, and the acquisition of chromatin accessibility downstream correlates with increased Dreg1 expression in ILC2 progenitors. Examining publicly available Tcf1 CUT&Run data, they find that Tcf1 was specifically bound to the accessible sites of the Dreg1 locus in early innate lymphoid progenitors. Finally, the syntenic region in the human genome contains two non-coding RNA genes with an expression pattern resembling mouse Dreg1.*
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> *The topic of the manuscript is interesting. However, there are various limitations that are summarized below.*

> *(1) The authors generated a new mouse model. The strategy should be better described, including the genetic background of the initially microinjected material. How many generations was the targeted offspring backcrossed to C57BL/6J?*

The mice were backcrossed for at least 2 generations to C57BL/6. This information is now included in the methods section.

> *(2) The data is obtained from mice in which the Dreg1 gene is deleted in all cells. A cell-intrinsic role of Dreg1 in ILC2 has not been demonstrated. It should be shown that Dreg1 is required in ILC2 and their progenitors.*

We now provide new mixed bone marrow irradiation chimera data that shows that the effect is intrinsic to *Dreg1*-deficient ILC2 cells (Figure 1F and Supplementary Figure 1E-G).

> *(3) The data on how Dreg1 contributes to the differentiation and or maintenance of ILC2 is not addressed at a very definitive level. Does Dreg1 affect Gata3 expression, mRNA stability, or turnover in ILC2? Previous work of the authors indicated that knockdown of Dreg1 does not affect Gata3 expression (PMID: 32970351).*

We have indeed shown that *Dreg1*-deficient ILC2P have reduced levels of Gata3 (Figure 2H) however we have not determined the exact mechanisms by which *Dreg1* controls ILC2 development.

> *(4) How Dreg1 exactly affects ILC2 differentiation remains unclear.*

We agree with the reviewer, however, this article is focused on the first description of the *Dreg1* knockout mice and the surprisingly specific effect on ILC2 development.

> ***Reviewer #2 (Recommendations for the authors):***
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> *(1) Relating to point 2 of public review:*
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> *It should be shown that Dreg1 is required in ILC2 and their progenitors. Mixed bone marrow chimeras would be an adequate strategy.*

We have now done this and clearly showed that the effect is intrinsic to *Dreg1*-deficient ILC2s.

> *(2) Relating to point 3 of public review:*
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> *Minimally, Gata3 expression should be analyzed in ILC2, ILC2P, and the ILC progenitors by qRT-PCR and antibody stain.*

We have indeed shown reduced Gata3 levels by antibody stain in Figure 2H.

> *(3) Relating to point 4 of public review:*
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> *The manuscript would benefit from additional data studying ILC2 differentiation in (competitive) adoptive transfer experiments or using in vitro differentiation assays.*

We have performed the mixed bone marrow chimera experiments which are testing the competitiveness of *Dreg1*-deficient bone barrow with control wildtype. In this case the WT ILC2s outcompeted the *Dreg1*-deficient ILC2s for the same niche.

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