**The genetic basis and cross-cell QTL trans-regulatory effects underly gut innate lymphoid cell regulation**

Miao Xu1,3,\*, Kirk Gosik1,\*, Yang Zhang4, Orr Ashenberg1, Chenhao Li1, Hongjun Li7, Kai Liu1, Leslie Gaffney1, Alok Jaiswal1, Gary Churchill8, Dan Gatti8, Kwangbom Choi8, Vivek Phillip8, Daniel Graham1, Heping Xu4,5,‡, Ramnik J. Xavier1,3,6,‡, Aviv Regev1,2,†,‡

1Broad Institute of Harvard and Massachusetts Institute of Technology, Cambridge, Massachusetts 02142, USA.

2Department of Biology, Howard Hughes Medical Institute, Massachusetts Institute of Technology, Cambridge, Massachusetts 02140, USA.

3Center for Computational and Integrative Biology, Massachusetts General Hospital, Boston, Massachusetts 02114, USA.

4Key Laboratory of Growth Regulation and Translational Research of Zhejiang Province, School of Life Sciences, Westlake University, Hangzhou 310024, Zhejiang, China.

5Westlake Laboratory of Life Sciences and Biomedicine; Laboratory of Systems Immunology, Institute of Basic Medical Sciences, Westlake Institute for Advanced Study, Hangzhou 310024, Zhejiang, China.

6Klarman Cell Observatory, Broad Institute of Harvard and MIT, Cambridge, Massachusetts 02142, USA

7MOE Key Laboratory of Bioinformatics, Center for Synthetic and Systems Biology, Bioinformatics Division, BNRist, Department of Automation, Tsinghua University; Beijing, China

8The Jackson laboratory, Boston, Massachusetts 02115, USA

\*Co-first author

†Present address: Genentech, 1 DNA Way, South San Francisco, CA, USA.

‡To whom correspondence should be addressed: [xuheping@westlake.edu.cn](mailto:xuheping@westlake.edu.cn) (HX), [xavier@molbio.mgh.harvard.edu](mailto:xavier@molbio.mgh.harvard.edu) (RJX), [aviv.regev.sc@gmail.com](mailto:aviv.regev.sc@gmail.com) (AR)

**Abstract**

Tissue function and regulatory complexity in homeostasis and disease arises from the coordinated function of multiple cell types and can differ between individuals in a population in part due to the impact of genetic variance. In many barrier tissues, such as the gut, lung or skin, innate lymphoid cells (ILCs) play critical roles in maintaining tissue homeostasis and immunity, but the full regulatory circuitry impacting ILCs intrinsically and extrinsically in the tissue remain largely unknown. To systematically elucidate such circuits in the small intestine, we performed single-cell RNA-seq (scRNA-seq) of 273370 ILCs from the gut of 274 diversity outbred (DO) mice with extensive natural genetic variations. Computational analysis identified quantitative trait loci (QTLs) and causal genes impacting ILCs at three levels: subtype-specific transcript abundance (in *cis*), ILC gene programs (in *trans*) and cell subset proportions, furthered by validating the pivotal role of *Rbpj* in regulating ILC3. All three classes of ILC QTLs display polygenic inheritance signature and broadly intersect with QTLs affecting peripheral cytokine levels in the mice. Remarkably, varied from the dogmatic recognition, *trans*-QTLs crossed cell type boundaries, such that variants in loci encoding genes expressed only in non-ILCs in the gut, such as epithelial cells, phagocytes and neurons, acting on the variation in ILC traits, unveiling causal cell-source to cell-target interactive genetic nodes and feedback in the tissue. Thus, we built up the framework enabling the broad dissection of the tissue specific genetic basis. We deciphered the tissue and cell type/state specific genetic basis and remarkably, discovered dominant QTL-mediated cross-cell *trans*-regulation underlie intestinal ILC biology.

**INTRODUCTION**

The complex molecular circuits within and across different types of tissue cells influence the cell-cell coordination, impacting tissue homeostasis, function and human diseases1. The genetic factors can lead to varied gene expression among individuals where many causative variants and associated genes wire the regulatory circuits and function in highly tissue and cell type specific manners2. The comprehension of genetic basis and principles in regulating tissue homeostasis in specific cell populations are largely unveiled, which calls for more inference of causality from genetic variations to the functional consequence of cellular phenotypic traits, as well as a systematic view of cell-cell coordination3. A case in point is innate lymphoid cells (ILCs), early responders to tissue perturbation, which are critical for sustaining homeostasis at barrier surfaces, a process which is largely directed by cell subset-specific cytokine production4–8. The development and responses of ILCs are fine-tuned to adapt to different signals in the intestine to confer regulatory or effector function9, through subset-specific gene expression, specialized gene programs, changes in ILC subsets, and intercellular crosstalk5. The physiological investigations5and SNP association studies10 have been implicating ILCs in tissue functional disorders and a broad range of human diseases such as infectious diseases, autoimmune diseases, neuronal disorders and so forth 11,12.

Recent genetics and genomics approaches have been powerfully applied to decipher gene function and circuits in both model systems and in humans. On the one hand, quantitative trait loci (QTL) studies, especially those applied to RNA13–15 and protein16 expression traits, have helped identify genetic variants and loci that impact the regulation of individual genes (in *cis*) and gene programs (in *trans*). However, with few exceptions16,17, these studies have mostly been conducted with bulk profiling, confounding signals from multiple cell types in one tissue, which both reduces power and limits the ability to distinguish cell intrinsic from cell extrinsic effects. On the other hand, single cell genomics, especially single cell RNA-seq (scRNA-seq) has been used to chart atlases of gene expression in complex tissues at single cell resolution18–20, and combined with Genome Wide Association Studies (GWAS) *post hoc*21to relate disease genes to the cell types in which they are expressed. However, few scRNA-seq studies, all of them in non-tissue settings17,22, such as iPSC lines or peripheral blood mononuclear cells (PBMCs), were conducted at a scale that allows direct genetic association *post hoc* integration, whereas *post hoc* analysis has less capacity for causal inference.

Performing scRNA-seq on tissue cells at the scale required for genetic association scale poses both experimental challenges, especially around accessing to and processing a large number of samples efficiently, coupled with genotype profiling, and computational ones, to leverage new opportunities for genetic association of cell type specific gene expression, gene programs and cell proportions, all recovered naturally by scRNA-seq. Establishing such approaches can benefit from appropriate model systems, which are both biologically meaningful, and reflect well the opportunities and challenges in the interface of genetics and single cell analysis. One compelling system is the diversity outbred (DO) mouse population, a heterogeneous stock derived from outbreeding of mice from eight founder strains (C57BL/6J, 129S/SvlmJ, PWK/Phj, NOD/ShiLtJ, A/J, NZO/HlLtJ, CAST/EiJ, WSB/EiJ)23,24, each of which has available whole genome sequence data. This mouse population presents balanced allele frequencies, simple population structure and extensive natural genetic variants within a relatively small sample size compared with human populations24. Importantly, it captures genetic diversity and predisposition at both the cellular and organismal levels, and thus serves as a more accurate model to elucidate genetic determinants of molecular and cellular phenotypic traits and disease susceptibility compared with standard laboratory inbred strains25.

Herein, we performed multiplexed scRNA-seq of high-quality 273370 ILCs from the small intestine of 274 outbred, genotyped DO mice, along with cytokine profiling in 261 DO mice, and developed a computational framework to identify genetic variants impacting ILC phenotypic traits including cell type specific gene expression (in *cis*), gene programs (in *trans*) and ILC proportions. With thousands of novel QTLs and QTL associated genes, we systematically wired the genetic regulatory circuits for intestine specific ILC trait regulation exerted by interconnected and polygenic QTL effects. We validated the role of one of *cis*-eQTL genes termed *Rbpj* in intestinal ILC3 regulation in vivo. Remarkably, different from previous dogmatic recognition, we found prevalent and dominant cross-cell QTL effects on *trans*-regulation for ILC trait consequence, revealing evolutionary genetic regulatory privilege. Those cross-cell QTL effects conferred by ILC QTL genes, which are not expressed in ILCs but expressed in cell types such as epithelium cell, phagocyte, neuron and so forth, were implicated to be essential in regulating both homeostasis and effective immunity such as infectious diseases, allergic diseases and broad inflammatory disorders26,27. Also, we opened up a new avenue with a general framework to identify causal tissue specific cellular genetic circuits in broader tissues and potential fundamental genetic regulation paradigms.

**RESULTS**

**Multiplexed scRNA-Seq in DO mice for QTL study of intestinal ILCs**

Using multiplexed scRNA-seq, we profiled the transcriptome of approximately 0.4 million total high-quality cells with 273370 ILCs from the small intestine of 274 outbred mice. We isolated the total ILC population of DO mice via fluorescence activated cell sorting (FACS) and performed scRNA-seq of ILCs in multiplexed pools (**Figure 1A, Methods**), which we subsequently demultiplexed based on genetic variants (**Methods**). In parallel, we genotyped each DO mouse with a GIGA-MUGA genotyping microarray28, capturing 143,179 SNPs across the mouse genome (**Figure S1A**). Following unsupervised clustering, we did *post hoc* annotation of high-quality total cells and ILC profiles based on known differentially expressed genes (DEGs) (**Methods, Figure S1B**). We then focused on canonical ILC subsets and ILC3 in different states: ILC1, ILC2, ILC3 (RORγthigh/RORγtlow) and ILC3-like LTi (CCR6high/CCR6low), hereafter referred to as “LTi” 6,29 (**Figure 1B**).

**Shared and specific eQTLs in ILC subsets**

We performed eQTL mapping in each of the four ILC subsets separately, to identify a total of 3,224 eQTLs (genome-wide significance *p*<0.05) affecting the level of 833 ILC genes, termed eGenes30 (**Figure 1C**). Of these, we found that 72.9% of eQTLs and 82.8% of eGenes were ILC subset-specific, while the rest were shared by two or more ILC subsets (**Figure** **1C**). We depicted the eQTL maps across the chromosome indicating the genomic loci associated with transcript abundance of eGenes for each ILC subset (left). 66.2% of the eQTLs were cis-eQTLs acting on eGenes within 1Mb or less, and the remainder 33.8% of eQTL acted distally 31–33 (**Figure** **1D**). Notably, we observed substantial trans association loci in chromosome 2 and 8 conferring eQTL effect on eGenes across multiple chromosomes, which might through the major regulators or transcriptional factors(Gata3 is located in chromosome 2)(**Figure 1D**). The majority of the cis- and trans-eQTLs were located in enhancers, indicating the regulatory acting manner of ILC eQTLs on gene expression34 (**Figure** **S1C**).

We then identified the genes harbored by ILC subset-specific eQTLs termed eQTL loci genes(**Methods, Supp Table**). Among these genes, for example, *Muc2*, encoding MUC2, located in the *trans*-eQTL region in chromosome 7 of ILC1, ILC3 and LTi, regulates the expression of eGenes *Stub1* and *Ift20* in ILC1(**Figure 1E**). In the intestine, MUC2 is the predominant gel-forming mucin that contributes to the formation of the mucus barrier, which functions to protect the intestinal barrier from microorganism and noxious substances, served as the first line of innate host defense 35. *Stub1* is an E3 ligase implicated as an important in regulation of gastric cancer36. *Ift20* encodes a transport protein IFT20 which can recruit ATG16L1 to promote autophagosome biogenesis in T cells while ATG16L1 is one of the most important multifunctional susceptible factors in inflammatory bowel disease(IBD) development37,38. Another genes is *Calca, in* a cis-eQTL region,encoding calcitonin gene-related peptide (CGRP)*,* is an important neuropeptide playing pivotal role in intestinal homeostasis through ILC211,39,40. We found that its QTL regulates the expression of eGenes in ILC2: *Ebp*, *Eif4a2*, *Actr10*, *Cd81*(**Figure 1F)**. Among them, *Ebp* encodes C/EBPδ, a pivotal transcriptional factor regulating cell proliferation and differentiation implicated in various pathological contexts41 including its role in protection against IR-induced intestinal injury and inhibition of ILC2 development in allergic inflammation42. eGene *Cd81* is a novel immunotherapeutic target for B cell lymphoma and it marks a mesenchymal population expansion and sustain stem cells by secretes the BMP antagonist in the small intestine 43. Thus, we identified the substantial number of cis- and trans-associations among cell type specific and shared eQTLs and established the ILC subtype specific molecular circuits from QTL and its loci gene to the eGenes.

**Variants in loci for key ILC and tissue homeostasis regulators, including *Fam21*, *Il20ra1* and *Rpbj*, affect the balance of ILC subsets**

The phenotypic imbalance of intra- or inter-ILC subsets in either local tissue or peripheral circulation are associated with the outcome of protective and pathogenic immune responses, such as inflammatory arthritis44, septic shock45and systemic lupus46, as well as disease susceptibility and severity47–49. For example, the ratio of ILC1 to ILC2 is correlated with lung function and COPD disease severity50. Immunotherapy tipped the balance IL-10+ILC2 / IL-5+IL13+ILC2 resulted in significant clinical response51. The ratio of IL22+IL-17+ILC3/ IFNγ+IL-17+ILC1 is associated with protective or pathogenic outcome in the intestinal tissues of patients52. We thus first measured the composition of ILC populations. We found that the composition evidently varied across DO mice: ILC3 and LTi were the most abundant intestinal ILCs, as expected53, individual DO mice showed particular enrichment of these or other ILC subsets, indicating the genetic impact on this trait(**Figure 2A**). We attempted to test for any genetic determinants for these variations in ILC subset proportions.

To this end, we mapped 2,738 “proportion QTLs”, defined as loci that affect the ratio of at least one pair of ILC subsets: ILC1/ILC2, ILC1/ILC3, ILC2/ILC3, ILC2/LTi, ILC3/LTi and ILC1/LTi (**Figure 2B; Figure S2B**). Another 277 QTLs underly the relative proportion of subsets within ILC3 (RORγthigh *vs*. RORγtlow) or within LTi (CCR6high *vs*. CCR6low), which represent the differential phenotypic and functional states of these cells54 (**Figure 2C**).

Likewise, we identified the putative causal genes located in the associated proportion QTL regions. Some of the these genes were implicated in key processes relevant to ILC regulation and tissue homeostasis. *Fam21*,which isin the QTL region associated with the ILC1/ILC2 ratio(**Figure S2C**)., encodes a known critical component regulating the endosomal localization of the WASH regulatory complex, which is essential in sustaining the intestinal NKp46+ ILC3 pool at homeostasis55. It was also implicated to be important in dendritic cell(DC) function in defending *Candida albicans*56. Consistently, we found that it is highly expressed in DCs and other lymphocytes including CD19+B cells, NK cells as well as present in ILC subsets(**Figure S2C**). The finding of *Fam21*:QTL confers significant effect on the ratio of ILC1/ILC2 suggested a new role of this gene in ILCs through either intrinsic or extrinsic regulatory manner. In another example, we found that *Il20ra* is in the QTL region associated with ILC3/LTi ratio(**Figure S2D**). The ratio of ILC3/LTi accounts for differential IL17 and IL-22 production levels in favor of proinflammatory or protective immune responses, respectively57. *Il20ra* polymorphisms are implicated in psoriasis development which was mainly driven by IL-23/IL-17 pro-inflammatory axis58. Through capture Hi C, *Il20ra* is characterized as a causal SNP gene regulating 6q23 over long-rang chromatin loops in T cells. 6q23 is one of the most important loci conferring risks for autoimmune diseases which are intimately associated with dysregulated inflammatory response 59. We found *Il20ra* is a QTL gene which is not only expressed in T cells but also highly expressed in ILC3 and LTi, hinting a node wherein ILC3/LTi derived *IL20ra* plays a role in tipping the balance of regulatory and effective inflammatory response in homeostatic state.

Most notably, the transcription factor (TF) *Rbpj* is located in a QTL region significantly associated with multiple ratio variations in terms of ILC2/ILC3, ILC2/LTi and CCR6highLTi/CCR6lowLTi(**Figure 2D**). *Rbpj* is one of the transcription factors in Notch signaling, which is implicated in ILC development and defense against infectious microorganisms 60–62. We observed a higher fraction of *Rpbj* expressing cells in ILC3s and LTis compared to other ILC subsets while all the relevant proportion trait variations are associated with ILC3 or LTi (Total ILC3) (**Figure 2D**). We reasoned RBPJ might play a role in regulating ILC3. To this end, we generated *Rorc*Cre*Rbpj*fl/fl conditional knock-outmice(*Rbpj*cKO) which specifically depletes *Rbpj* in total *Rorc*-expressing ILC3s(ILC3 and LTi) and compared to *Rorc*Cre*Rbpj*+/+ mice (*Rbpj*WT) (**Figure 2E**). We isolated the total intestinal CD45+ cells from the small intestine and the ILC subsets are distinguished according to defined markers as: total ILCs(live CD45+Lin-CD90.2+), total ILC3s(RORγt+ILC3s: ILC3 and LTi), RORγt+NKP46+CCR6-ILC3s(Conventional ILC3), RORγt+NKP46-CCR6+ILC3s(ILC3-like LTi). We found that, compared with *Rbpj*WT mice, the deficiency of *Rbpj* led to significantly decreased ratio of total ILCs, total ILC3 and conventionalILC3 in CD45+ cells (**Figure 2F, 2G; Figure S2E**). The ratio of total ILC3 and conventional ILC3 in total ILCs and the percentage of conventional ILC3 was decreased in total ILC3 were also significantly decreased in *Rbpj*cKOmice compared with *Rbpj*WTmice (**Figure 2G; Figure S2E, S2F**). We then measured the actual relative proportion changes and found the evident alterations for all three kinds of relative proportions: CCR6+ILC3/CCR6-ILC3, ILC2/ILC3 and ILC2/LTi (CCR6+ILC3), displaying the consistency with the computational predictions (**Figure 2D, 2H-2J**). In an attempt to determine the impact of *Rbpj* on cytokine production capacity, we restimulated the ILCs with PMA and ionomycin followed by intracellular cytokine detection. Interestingly, compared with ILC3s in *Rbpj*WTmice, *Rbpj*cKO mice derived ILC3s conferred significantly increased IL-17A and IL-22 production levels(**Figure 2K, Figure S2G**). Thus, RBPJ, as a proportion QTL gene, is sufficient in regulating the proper ILC composition in the intestine, as well as the cytokine production levels of total ILC3. Herein, we deciphered the genetic basis underlying the variations in proportion in major ILC subsets and their further states and identified the variants and associated gene regulators, as exemplified by *Fam21*, *Il20ra* and *Rpbj*, affecting the balance of ILC subsets with key ILC and tissue functional consequences.

**QTLs impacting ILC cross-cell gene topics harbor regulators of intestinal function**

Specific gene program or the combination of gene programs can characterize cell states and detangle context-dependent QTL effect63.Given the substantial number of cis- and trans-associations among cell type specific and shared eQTLs and, we tested for genetic variation associated with differences in gene programs, which can capture both cell subset distinctions, variations within a cell subset, and shared processes64,65. To achieve this, we adopted unsupervised “topic modeling” 64which largely increases the capacity to capture the fluid functional states and transitional gene programs among the cell population and identify the gene programs as a quantitative trait(**Figure 3A**). We identified 20 cross-cell distinct programs (“topics”) spanning either a single canonical ILC subset or cross-cell clusters (**Figure 3B; Figure S3A, Supple**). Among cross-cell topics, for example, the topic 0, spans 85 high scoring genes including *Ifngr1, Ncr1, Junb, Il2rb, Ccl5,Ccl3,Ccl4* and so forth, are highly expressed in ILC1s, and enriched in genes with functions associated with ILC1s and related processes, such as the response to IFNγ, or NK cell-associated immune responses66(**Figure 3C**). Topic 11, scores highly in ILC2s and is correlated with the expression of its signature TF *Gata3* and of *Calca* (**Figure 3B,3D**). Topics 3, 8, 12 and 16 each score highly in a different portion of ILC3s and/or LTis, the high scoring genes of which are overlapped in largely differential sets of biological processes. Topic 3 score highly in CCR6high LTi while topic 16 score highly in CCR6lowLTi. The high scoring genes are either enriched in similar processes such as myeloid cell differentiation and viral gene expression or distinct biological processes of which high scoring genes in CCR6lowLTi topic 16 are overlapped in intrinsic apoptotic pathways and response to IL-4 or hyperoxia while the genes in CCR6highLTi topic 3 are enriched in functions such as myeloid cell homeostasis, positive regulation of NF-κB signaling pathway, monocyte differentiation, cellular response to TGF-β and so forth, highlighting the distinct functional states even within the same classic ILC subsets (**Figure 3C**).

We then performed QTL mapping to identify QTLs accounting for the variation of different cross-cell topics, termed “topic QTL”. We found the topic QTLs for 14 topics and these QTLs significantly act on at least one of 14 ILC cross-cell topic variations while some of the QTLs exert effect on multiple topic QTL modeling (**Figure 3E, Figure S3B**). The genes harbored by topic QTLs are also identified and a slew of these genes are shared for different topic modeling. For example, the topic QTL genes including *Sstr4*, *Thbd*, *Cst12* and *Cst8 and so forth* are either solely associated with topic 1 or topic 3 modeling or shared for exerting the effect on the variation of both topic 1 and topic 3(**Figure 3E**). Among the overlapping genes, *Sstr4*encodes a receptor for regulatory peptide somatostatin(SOM) that activates G protein-coupled receptors. In the intestine, SSTR4 can regulate the expression of inflammatory peptides including inhibition of CGRP and SP as well as the nociception. It can also modulate the expression of SOM, SSTRs, and neuron innervation. It involved in both pro- and anti-inflammatory peptides expression in the murine small intestine 67. Thus, through finely defined topic modeling for ILC populations and QTL mapping, we elucidated topic QTL effect on shaping ILC gene programs and identified the QTLs harboring regulators of intestinal function

**Intra- and across-trait category of intersected QTLs synergistically regulating ILC traits and serum cytokine levels**

Given the observation of shared QTL harboring regulators of critical physiological and pathological functions(**Figure 3**), we next systematically examined the QTL intersection in regulating ILC traits. We found substantial QTL intersections both within a specific trait category and across categories (**Figure** **4A-4C)**. Specifically, there are XX, XX and XX QTLs shared for effects on ILC subset expression, proportion and topic modeling. There are also XX, XX and XX QTLs shared for exerting QTL effect across ILC subset expression, proportion and topic modeling. These QTL intersections hint at the causal mechanism for how a single QTL contribute to the ILC phenotypic traits. For example, *Il22ra1*, is in the QTL regulating ILC3 eGene expression and also conferring effect on the relative proportion of ILC3/ILC2. While ILC3 is an essential producer of IL-22 and IL-22RA1 is expressed in intestinal epithelium68, our results indicates a reciprocal feedback mediated by IL-22 receptor IL22RA1 on ILC3 regulation regarding gene expression and ILC3/ILC2 axis(**Figure 4D**).

Peripheral serum cytokine levels provide practical opportunities in clinical diagnosis for various diseases including intestinal inflammation and diseases and can be largely contributed by circulating T cells and ILCs and their crosstalk69,70. Circulating ILCs are homing to tissues including intestine under certain circumstances, affecting the intestinal homeostasis. Tissue-resident intestinal ILCs serve as predictors of diseases while it is hard to characterize them in the tissue directly. Thus, we asked if there are genetic links(QTL intersections) between peripheral serum cytokine levels and intestinal ILC traits.

To test this, we collected the serum from each of 262 DO mice at homeostasis. We performed a large-scale detection for an array of cytokines for DO mice, including IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-17F, IL-22, IFN-γ and TNF-α, which are associated with ILC effector and regulatory response in different contexts4,7,71. Among the DO mouse population, the levels of each cytokine levels were highly varied suggesting the genetic impacts on serum cytokine levels(**Figure 4D; Figure S4A**). Likewise, we performed genome-wide QTL mapping and identified a total of 141 genetic variants exerting QTL effects on the varied cytokine levels, which we termed “cytokine QTL” (**Figure 4F; Figure S4B**). We then identified the putative causal genes in these QTL regions which are either known genes relevant to certain cytokine production or novel genes whose function is then determined. For example, *Smad2* is in the QTL exerting dual effect on both levels of IL-9 and IL-17F, which are signature cytokines of Th17 cells, correlated with its role in regulating Th17 differentiation and function72–74(**Figure 4G**)

To further elucidate the genetic link between peripheral cytokine levels and intestinal ILC traits. We performed QTL intersection analysis to wire the interconnected genetic links between ILC traits and serum cytokine levels. Importantly, 25 of the 141 cytokine QTLs overlapped with ILC QTLs of either subset-specific transcript abundance (15 QTLs), programs (topics) (5 QTLs) or cell proportion (14 QTLs) (**Figure** **4H, 4I; Figure S4C, 4D)**. For example, a QTL in the *Bcl2* locus is associated both with variation in serum IL-5 level and with topic 19 shared by ILC1, ILC2, and ILC3(high scoring genes: *Bcl2*, *Ptpn22*, *Il21r*, *Junb*, etc). *Bcl2* was previously implicated in regulating both ILC2 survival and IL-5 production through ICOSL-ICOS signaling in ILC275 . It is also expressed in goblet cell as a critical mediator of intestinal transformation following stem cell mutations76(**Figure 4J**). In another example, we found that a QTL in the *Ptpn5* locus is associated with variation in both *(eGene)*expression in ILC1 and serum levels of ILC1 signature cytokine IFNγ[46](https://app.readcube.com/library/d2d03881-4f35-4171-bc9f-362ae7c2b2a9/all?uuid=015888134730278947&item_ids=d2d03881-4f35-4171-bc9f-362ae7c2b2a9:b0e00f56-0cae-49bc-ba34-845ead2efdc7). *Ptpn5* encoding STEP which was implicated to regulate synaptic junction and neuronal cell survival in several neuronal disorders77,78. It is essential in apoptosis in *Bak*-mediated apoptosis in mouse embryonic fibroblasts79 and also involved in the differentiation and activation of γδ T cells which are the producers of IL-17A and IFNγ80(**Figure 4I, 4K**).

Thus, we identified intersected QTLs forboth intestinal ILC traits and peripheral cytokine levels and wired up genetic links between these traits, suggesting the composite functional consequences affected by QTLs.

**ILC traits are controlled by a polygenic architecture, enriched with genes for polygenic pathways**

Most phenotypic variation in complex traits, including common disease, is explained by polygenic inheritance, caused by the joint contribution of a number of independently acting or interacting polymorphic genes, with a relatively modest effect1,81. In QTL intersections we detected many individual QTL can affect multiple trait variations(**Figure 4**). We also observed the broad existing pattern in which multiple QTLs exerting effect on individual category of trait(**Figure 4A-4C**; **Horizontal sets**). Moreover, as mentioned above, topic 8 modeling is associated with 3 QTLs located in chromosome 3, 5, and 11(**Figure 3D**). By calculating founder strain QTL effect coefficients inferred from the mapping model, we also found these QTL can exert effect on topic 8 in different founder strains with different extent of QTL effect wherein NOD strain and WSB strain showed most significant association with this topic(**Figure 5A**).

To systematically characterize the QTLs potentially synergized for the trait regulation, we assessed polygenicity of QTLs in regulating ILCs. Generally, ILC phenotypic traits – including transcript abundance, subset proportions and gene programs – were typically polygenic, associated with 3.7, 75, 2.6 QTLs on average, whereas each peripheral cytokine level was associated with few QTLs (1.7 on average) (**Figure 5B**). While ILC cell type specific expression are either associated with individual QTL or multiple QTLs, notably, all proportion and program traits were associated with multiple QTLs(**Figure S5A**).

We also found characterized the polygenic variants and found that most of them are non-synonymous and located in the non-coding region of the genome (**Figure 5C**).

To further assess how these polygenic QTLs contribute to trait. We performed GO analysis for the polygenic QTL associated genes. We found that some of the genes in QTLs associated with the same trait or same categories of trait are enriched in trait-relevant and novel processes, suggesting convergence to causal functional pathways (**Figure 5D**). The genes in QTL accounting for ILC proportions are mostly enriched in biological processes such as synaptic vesicle cycle, neuron transmitter secretion and their regulation while these processes are well connected (**Figure 5E**). The genes in QTL conferring effect on proportion of ILC2/ILC3 are enriched in processes such as bile acid and bio salt transport, negative regulation of cell projection, in addition to the synaptic and neuron transmitter secretion(**Figure 5F**). Notably, the genes in QTL exerting effect on LTi state regarding CCR6high/CCR6low are mostly associated with the processes of potassium ion transport which are widely implicated in physiological and pathological function in intestine biology82,83(**Figure 5G**).

In an attempt to identify QTLs acting on the key transcriptional factors(TFs) for ILC subsets, including *Tbx21*(ILC1), *Gata3*(ILC2), *Rorc*(ILC3 and LTi) 7, we did not find individual QTL significantly associated with the expression of these genes but an array of genetic alleles. We quantified the polygenic risk score(PRS) for these traits. Notably, the polygenic score contributed by genetic alleles to the expression level of lineage-specific TF gene produced continuous, normally-distributed and evident lineage-specific patterns(**Figure 5H**). Specifically, for example, only in ILC1 context, the DO mice bearing the polygenic alleles can positively affect *Tbx21* expression(high PRS). Of note, the collective effect of polygenic alleles positively affect *Rorc* expression are in LTi context rather than conventional ILC3. Corresponding to previous studies indicating *Ncr1* is commonly used marker distinguish classic ILC3 from LTi especially in the intestine84,85, we found that in ILC3 context, the polygenic alleles showed more specific effect for *Ncr1* expression than *Rorc* (**Figure 5H**).

The heritability from genotype to phenotypic traits is multifactorial, especially for the mouse with outbreeding and traits affected by polygenic alleles/QTLs. We leveraged the founder strain mice to profile a total of 80,000 intestinal ILCs from 32 founder strain mice(4 mice for each founder strain), and estimated the heritability of variation in transcript affected by individual QTL. We compared the founder strain QTL effect coefficients in DO mice at variant loci (predicted) to the actual effects(positive/negative) in founder strain mice (observed). As predicted, genes whose expression is associated with *cis*-eQTLs had overall higher correlation between predicted and observed expression than those with *trans*-eQTL associations (**Figure** **S5B**, STATS, P Value). As an example, *Hopx* is a eGene affected by 1 QTLs, inferred from DO mice.The corresponding *cis*-eQTL regionpredicted positive correlation with *Hopx* expression among founder strain mice: A/J, C57BL/6J, CAST/EiJ, NZO/HILtJ, and PWK/PhJ (**Figure S5C**).

Together, we illuminated the genetic architecture wired predominantly by polygenic QTLs harboring genes enriched

**Cross-cell *trans*-regulation of ILC traits by QTL genes reveals epithelial-, phagocytes- and neuron-ILC circuits**

The intestinal ILCsare in spatial neighborhood of a number of intestinal cells such as epithelium cells, enterocyte neurons, other sorts of lymphocytes and so forth, and these cell types are frequently interacted to maintain the homeostasis and immunity5,7. We noticed a range of the *trans*-QTLs affecting variation in ILC traits carried genes that were not/merely themselves expressed in ILCs(e.g. eQTL *Muc2*, proportion QTL *Fam21*), hint the cross-cell type QTL effects for ILC regulation in the intestinal tissue. A number of other instances of intercellular trans-regulation circuits are recovered by our QTL analysis, where genes with non-ILC, cell type-specific expression reside in QTLs that causally affect ILC traits, and, in some cases, the ILC traits in turn impact the source cell of the QTL gene, closing a tissue feedback loop. For example, *Sox9*, encoding an intestinal crypt TF expressed in intestinal epithelium cells, regulates Paneth cell regeneration and intestinal homeostasis86,87. It is located in the QTL impacting variation in the ILC3/LTi topic 8, which is enriched for leukocyte differentiation and immune response regulation(**Figure 3C**), with the high scoring genes including *Rorc*, *Jun*, *Fosl2, Cd69*, *Il-22*, *Rora*, *Il7r, etc*. In our data, as expected, *Sox9* is expressed in enterocytes rather than ILCs (**Figure 6B)**.This suggests that the *trans*-QTL reflects cross-cell type action through an intercellular regulatory circuit: enterocyte-specific *Sox9* influencing a gene program in a subset of ILC3s. Moreover, the program includes genes, such as *Il-22*, as well as *Rorc* and *Rora* which are key regulators for Th17 response, that in turn are known to affect enterocyte function88,89, thus closing a putative feedback loop in the tissue modulating intestinal homeostasis: *Sox9* (enterocyte)- (ILC3/LTi) Topic 8- IL22(Th17)-enterocyte(**Figure 6C**)73,89,90. In another example, IL-25, secreted from tuft cells in the small intestine, has been shown to regulate intestinal allergic response and homeostasis through ILC291,92. Corresponding to the previous studies, we found it is not expressed in ILC2 but located in the QTL associated with variation in ILC2 expressed genes: *Kctd12*, *Xlr4c* and *Il2ra*. We also found IL-25 QTL region associated with the relative proportion of [RORγt](https://app.readcube.com/library/?style=Nature)highILC3/[RORγt](https://app.readcube.com/library/?style=Nature)lowILC3 while IL-25 was implicated to play hierarchical role in ILC development and ILC2 could directly impact ILC1/ILC3 activity in lung mucosae 93 (**Figure S6A, 6B**).

We also found that *Ccl17*, is in the QTL impacting the proportion of [RORγt](https://app.readcube.com/library/?style=Nature)highILC3/[RORγt](https://app.readcube.com/library/?style=Nature)lowILC3 and the expression of gene *Cd48*, *Frmd4b in* ILC2 and ILC3 (**Figure 6D**). CCL17 is implicated to be secreted by phagocytes such as dendritic cells(DCs), macrophages and monocytes to mediate tissue inflammation such as arthritis, EAE, Multiple Sclerosis as well as acting as a novel therapeutic target for pathological cardiac hypertrophy through DC-CCL17-Th2 axis-IL-4 axis94–96. *Ccl17* is not expressed in ILCs but its receptor gene *Ccr4* is selectively expressed in ILC2(**Figure 6E**). This suggests a putative direct circuits of phagocyte-CCL17- CCR4-*Cd48*, *Frmd4b expression in* ILC2 and also an indirect circuit in which CCL17 regulates ILC3 states accounting for inflammatory regulation(**Figure 6F**).

The cross-cell QTLs also reflect known neuron-ILC interactions, where neuronally-expressed neuropeptides: neuromedin U (NMU), substance P (SP) and vasoactive intestinal peptide (VIP) are encoded by genes harbored by QTLs associated with differential ILC traits 11. Specifically, the *NMU* gene is located in a locus acting on proportion of ILC1/LTi and ILC2/LTi, ILC2-specific *Lman2* expression, as well as topic 8 modeling (**Figure 6G**). The homeostatic node of QTL-NMU-ILC2 related proportion and eGene *Lman2* expression in ILC2 adds up to a validated neuron-NMU-ILC2 axis in small intestine40,97, where *Nmu*, highly expressed by cholinergic enteric neurons, regulates ILC2 response to worm infection66. Likewise, we checked the expression of *Nmu* and *Nmur1*, the receptor gene of *Nmu. As expected,* NMU is not expressed in any of ILC subsetswhile *Nmur1* is not only expressed in ILC2, but modestly expressed in ILC3, LTi and ILC1, indicating the potential role of *Nmu* in regulating multiple ILC phenotypic traits(**Figure 6H, 6I**).

Substance P (SP) is a neuropeptide and neuromodulator encoded by *Tac1*. SP can act on mast cells to promote the release of inﬂammatory mediators in the skin26. It can also regulate smooth muscle contractility, epithelial ion transport, vascular permeability, and immune function in the gastrointestinal tract in IBD pathophysiology development98–100. We found it is located in QTLs affecting the relative proportion of CCR6high/CCR6lowLTi relevant to IL-17 and IL-22 expression levels 66, as well as the expression of eGenes *Sec61b and Rpn2 while Rpn2 is also a ILC2 cis-eQTL gene and was implicated in colon carcinoma cell proliferation and apoptosis*101,102, indicating a SP-ILC(*Rpn2*, *Sec61b*) axis potentially involved in intestinal inflammation development(**Figure S6C, 6D**).

Neuropeptide VIP is in the QTL regulating relative proportion of [RORγt](https://app.readcube.com/library/?style=Nature)highILC3/[RORγt](https://app.readcube.com/library/?style=Nature)lowILC3(**Figure S6E, 6F**). Of interest, *vipr2*, the VIP receptor gene, is in a QTL also affecting the proportion of ILC3/LTi(**Figure S6G, H**). These associations correspond to studies showing that VIP, binding to VIPR2 on ILC3s, stimulates the production of IL-22, critical in the maintenance of immune homeostasis and metabolic processes at intestinal barrier[60,61](https://app.readcube.com/library/d2d03881-4f35-4171-bc9f-362ae7c2b2a9/all?uuid=670557411342924&item_ids=d2d03881-4f35-4171-bc9f-362ae7c2b2a9:f229fe22-29cb-4ea5-be19-7cdb220e7369,d2d03881-4f35-4171-bc9f-362ae7c2b2a9:4f21de30-59b3-43a4-91b5-eaf65e054ebe). Our findings confirm and suggest the QTL acting manner(ILC3/LTi balance) in the VIP-VIPR2-ILC3 circuit.

Besides our sc-RNA seq data in DO mice, we also checked the expression across distinct single cell RNA seq datasets independently generated: Small intestinal epithelium single cell18; mouse enteric nervous system103, as well as in the cells of the human intestinal tract atlas104. These data shows the consistent non-ILC expression pattern for Sox9, Il25, Ccl17, Calca, Vip, Tac1 as in our DO mice dataset(**Figure 6J**).

Altogether, our data indicated extrinsic cross-cell *trans*-regulation of ILC traits by QTL genes reveals neuron-, phagocyte- and epithelial-ILC circuits.

**QTLs with non-ILC genes are prevalent *trans*-regulators of ILC genes, programs and proportions**

The previous dogmatic recognition for how QTL or other genetic markers acting on the cellular traits are usually in intrinsic manners, mapping variant effect onto corresponding cell types21.

We have observed a lot of cases() where genes in QTL exerting polygenic effect on single or multiple ILC traits through cross-cell trans-regulation. Part of the genes in these QTLs which were implicated in various essential cross-cell regulatory circuits() can be finely reflected in our study through QTL mapping(**Figure 6**).

We then systematically examine if ILC QTLs prevalently harboring non-ILC expressing genes to mediate cross-cell regulation(QTL intrinsic effect on regulating ILC traits through cross-cell trans-regulation), excluding the possibility that the genes exemplified above are occasionally located in QTL regions we identified.

We first examined the expression of QTL genes in ILC populations. We found that less than 20% of the ILC QTLs haring genes for each of the traits (expression, cell proportion or gene topics) were expressed in ILC populations (**Figure 7A**). Specifically, [break down numbers for cis eQTLs of eGenes, trans eQTLs, proportions and topics]

To further characterize the cross-cell trans-regulation, we attempted to scrutinize the expression pattern of QTL genes in different cell types through gene module enrichment(**Methods**). Specifically, we examined the expression of the genes in the ZZ% of QTLs that did not harbor an ILC-expressed gene in several broader contexts: (1) DO mice sc-RNA seq data with other cell types. (2) Small intestine immune cells from Lamina propria and Peyer’s patch at homeostasis64. (3) Small intestinal epithelium single cell18; (4)ENS

In addition to the single cell RNA seq datasets generated in our lab: (**2**) A pan-tissue atlas of the mouse (Tabula muris)19; and (**3**) Cells of the human intestinal tract across space and time104; as well as(4) Human and mouse enteric nervous system103.

The examination of the expression in dataset of small intestine immune cells from Lamina propria(LP) and Peyer’s patch(PP) at homeostasis64 (**Figure 7B**) showed that in LP and PP,

Across epithelium cell types 18, non-ILC expressed genes were expressed in a variety of epithelium cells in the small intestine, and particularly enriched in enteroendocrine, goblet and Paneth cells. The expression pattern for non-ILC expressed genes in the QTL for topic modeling was more diverse, in which they were enriched in enteroendocrine, Paneth cells, stem cells and topic specific expression enrichment patterns(e.g. Tuft cell derived QTL gene affecting Topic 10, 11, 13 and 19 modeling) (**Figure 7C**).

The scrutinize of expression in a pan-tissue atlas of the mouse (Tabula Muris)19 shows the tissue specific enrichment pattern of high enrichment of genes in the eQTLs and proportion QTLs in the bone marrow, thymus, spleen pancreas, tongue and fat(**Figure 7D)**

Together, these findings reveal the QTLs with non-ILC genes, widely derived from epithelium/endothelium cells, phagocytes, neurons and lymphocytes, are prevalent trans-regulators, of ILC genes, programs and proportions.

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