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### Single-Cell RNA Sequencing of Blood and Ileal T Cells From Patients With Crohn's Disease Reveals Tissue-Specific Characteristics and Drug Targets

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#### Keywords

Inflammatory Bowel Disease; Genome-wide Association Study; Immune Response; Genetics

Crohn's disease (CD) is a chronic inflammatory disease predominantly affecting the terminal ileum. The ~200 CD-risk loci identified by genome-wide association studies are enriched for genes involved in T-cell signaling, highlighting the importance of T cells in CD pathology.<sup>1,2</sup> It is crucial to study T cells in their disease-relevant context, namely the

Conflicts of interest

There are no (potential) conflicts of interest to declare.

Writing assistance

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<sup>\*</sup>Authors share co-first authorship.

Author contributions

Werna T. Uniken Venema and Michiel D. Voskuil contributed equally to this work and are shared first authors. Werna T. Uniken Venema, Michiel D. Voskuil, and Eleonora A. Festen participated in the conception, design and coordination of the study. Gerard Dijkstra, Rinse K. Weersma, and Eleonora A. Festen recruited patients. Werna T. Uniken Venema, Michiel D. Voskuil, and Bernadien H. Jansen performed the laboratory experiments. Werna T. Uniken Venema, Michiel D. Voskuil, Gerben van der Vries, Arnau Vich Vila, and Eleonora A. Festen performed the data analyses and data interpretation. Bana Jabri, Klaas Nico Faber, Gerard Dijkstra, Ramnik J. Xavier, Cisca Wijmenga, and Daniel B. Graham provided support with data interpretation. All authors assisted in the writing and reviewing of the manuscript and approved of sending it out for publication.

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Supplementary Material

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intestinal mucosa. Although human single-cell atlases are in development,<sup>3</sup> location-and disease-specific single-cell RNA sequencing (scRNAseq) datasets are still scarce. In this study, we used scRNAseq of disease-relevant cells to examine pathomechanisms and identify potential drug targets on a cellular level in CD.

#### Methods

We performed flow cytometry and scRNAseq of 5292 CD3-positive T lymphocytes isolated from peripheral blood (PBLs) and ileal biopsy specimens from 3 patients with CD with mild to moderate disease activity (Figure 1, step 1). Biopsy specimens were dissociated and separated into intraepithelial T lymphocytes (IELs) and lamina propria T lymphocytes (LPLs).<sup>4</sup> Then, we integrated T-cell transcriptomes with genome-wide association study CD-risk loci and drug-target identification resources. Through a literature search and online database analysis, we identified 179 CD-risk genes and 2712 drug-target genes that we aligned to differentially expressed genes. Then, we selected genes encoding proteins targeted by drugs currently available for (clinical trials in) humans (Supplementary Methods).

#### Results

After quality control, 4070 T cells remained for analysis. These cells expressed 966 genes per cell, on average, and 41,134 distinct genes in total (Figure 1, step 2). IELs, LPLs, and PBLs showed markedly different expression profiles (Supplementary Figure 1 and Supplementary Table 1).

Unsupervised clustering of scRNAseq data identified 6 distinct T-cell types that have different distributions in IELs, LPLs, and PBLs: cytotoxic T lymphocytes (CTLs) dominate IELs, quiescent T cells dominate the PBL reservoir, and T-helper 17 (Th17) cells dominate LPLs (Figure 1, step 3). In peripheral blood, T-regulatory (Treg) cells intermixed with effector T cells (effector/Treg cells) and quiescent T cells (Treg/quiescent cells). Treg/ quiescent cells also were present among IELs and LPLs. Among IELs and LPLs, we identified a cluster of cells of a previously undefined cell type characterized by expression of *CD3* and *REG1A/B* and confirmed this with immunofluorescence staining. All T-cell subtypes were present in all patients.

Strikingly, all cell-subtype clusters consisted of epitopic  $CD8a\beta$ -positive and -negative cells. Moreover, *CD8A* and *CD8B* transcripts were expressed in epitopic  $CD8a\beta$ -positive and - negative cells, which was confirmed in a publicly available naïve  $CD4^+$  T-cell scRNAseq dataset.<sup>5</sup>

Using permutation analysis, we found that IELs and LPLs expressed significantly more CDrisk genes than expected by chance (P = .00389 and P < .00001, respectively), suggesting that cells within these compartments play a role in CD inflammation.<sup>2</sup> PBLs were not enriched for CD-risk gene expression (P = .45954). Th17 cells showed the largest number of over-expressed CD-risk genes and were most specifically enriched for CD-risk gene expression (P < .00001). Mucosal CTL and Treg/quiescent and peripheral blood effector/ Treg cells also were significantly enriched for CD-risk gene expression (P = .0278, P = .02477, and P = .00081, respectively).

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We investigated which drug targets are expressed by Th17 cells and CTLs, cell types with well-characterized expression signatures that play a central role in CD pathogenesis (Table 1). Th17 cells showed up-regulation of *IL17A*, whose gene product is targeted by secukinumab. *ITGAE*, whose gene product is targeted by etrolizumab, was upregulated in mucosal CTLs. *S1PR5*, up-regulated in peripheral blood CTLs, is a known drug target for ozanimod. Potential targets for drug repositioning include *PDE4D* in mucosal Th17 cells, a target for apremilast, under investigation for treatment of ulcerative colitis; *ITGB2* in peripheral blood CTLs, a target for lifitegrast, approved for keratoconjunctivitis sicca; and *ALOX5AP* in mucosal CTLs, a target for fiboflapon, under investigation for asthma.

#### Discussion

We have demonstrated that multiple ileal mucosal T-cell subtypes and 1 peripheral blood Tcell subtype from patients with CD are enriched for CD-risk gene expression. T-cell subtypes known to be involved in CD pathogenesis provide promising targets for future cell type-specific therapies in patients with CD. A limitation of our study is the small sample, which might decrease the amount of variation covered. However, most cell type-specific gene expression signatures remained after correcting for inter-individual differences. Because location-and disease-specific scRNAseq data are still limited, detailed datasets like ours are an important reference for furthering our understanding of the molecular processes leading to health and disease and identifying potential targets for drug development.

#### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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#### Abbreviations used in this paper:

CD	Crohn's disease

CTL cytotoxic T lymphocyte

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IEL	intraepithelial T lymphocyte
LPL	lamina propria T lymphocyte
PBL	peripheral blood T lymphocyte
scRNAseq	single-cell RNA sequencing
Th17	T-helper 17 cell
Treg	T-regulatory cell

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#### Figure 1.

Experimental flowchart. EDTA and collagenase are treatments used to isolate IELs and LPLs, respectively. T-cell subtype characterization was performed as described in the Supplementary Methods. The number of epitopic  $CD8\alpha\beta$ -positive T cells measured by flow cytometry is presented within brackets. FACS, fluorescence-activated cell sorting; TCR, T-cell receptor.

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# Table 1.

Overexpression of CD-Risk Genes and Genes Encoding Potential Drug Targets in Ileal Mucosal Th17 Cells and CTLs

GeneDrug or compoundGeneDrug or compoundGeneICD-risk gene $CC220$ Chemoattractant for various immune cells $CTSW$ Regulation of T-cell cytolytic activity $PLC02$ system resultsDVAJDHHeat shock protein, involved in adaptive-imaate $DTW$ Regulation of T-cell cytolytic activity $PLC02$ system results $DVAJDH$ Heat shock protein, involved in adaptive-imaate $DTW$ $APRN22$ NegulationNegulation $BTAT$ $DVAJDH$ Regulation of mucosal Th17 cell $PTRC6$ Apptosis regulation $SOCS1$ Cytokine- regulation $BTAT$ Regulation of mucosal Th17 cell $PTRC7$ T-cell antigen receptor signaling $PTRV22$ Negulation $BTAT$ Regulation of mucosal Th17 cell $PTRC6$ $T-rell antigen receptor signalingPTRV22NegulationBTATRegulationDTTRC7T-cell antigen receptor signalingTTATA2NegulationBTATSectivitianaS1PR6OzaimodCC26T-rellBTATATATATATATATATATATATATATATATATATATA$			Mucosal Th17 cells		Peripheral blood CTL		Mucosal CTL
CD-risk geneCCL20Chemoattractant for various immune cellsCTSWRegulation of T-cell cytolytic activity $LCC2$ TanawanDMAIB4Heat shock protein, involved in protein $LSPI$ Adhesion and transendothelial $PTPN2$ Negative $DMAIB4$ Heat shock protein, involved in protein $LSPI$ Adhesion and transendothelial $PTPN2$ Negative $DMAIB4$ Heat shock protein, involved in adaptive-innate $PRKCB$ Apoptosis regulation $SOCS1$ Negative $DMAP24$ Regulation $PTPR2$ Regulation $SOCS1$ $SOCS1$ Cophine- $DAP248$ Regulation of mucosal Th17 cell $PTPR2$ Terell antigen receptor signaling $SOCS1$ Cophine- $DAP249$ IL74Regulation of mucosal Th17 cell $PTPR2$ Terell antigen receptor signaling $SOCS1$ Cophine- $DAP240$ IL74Sectimate $PTR2$ Crealingtigen receptor signaling $CCC5$ $DOCS1$ $DOCS1$ $DOCS1$ $DAP240$ IL74Sectimate $STR2$ $SOCS3$ $CCC5$ $DOCS1$ $DOCS2$ $DOCS1$ $DOCS1$ $DOC500$ $DOC500$ $DOC500$ $DOCS2$ $DOC500$		Gene	Drug or compound	Gene	Drug or compound	Gene	Drug or compound
D'AJB4Heat shock protein, involved in protein $LSP1$ Adhesion and transendothelial $PTPV22$ Negative positive regulation $IFVG$ Cytokine, involved in adaptive-innate $PKCB$ Apoptosis regulation $SOCSI$ Cytokine- regulation $IFVd$ Equation of nucosal Th17 cell $PTPC$ T-cell antigen receptor signaling $SOCSI$ Cytokine- regulation $IF74$ Regulation of nucosal Th17 cell $PTPC$ T-cell antigen receptor signaling $SOCSI$ Cytokine- regulation $IF74$ Regulation of nucosal Th17 cell $PTPC$ T-cell antigen receptor signaling $SOCSI$ Cytokine- regulation $IF74$ Secukinumaby $ITF7A$ Secukinumaby $ITF7A$ T-cell antigen receptor signaling $SOCSI$ Cytokine- regulation $IR74$ Secukinumaby $ITF7A$ Secukinumaby $ITF7A$ $ITC7A$ $ITC7A$ $ITC4A$ $ITC4A$ $ITC7A$ Secukinumaby $SIPA3$ IgC class mAbs <sup>d</sup> $ITC7A$ $ITC4A$ $ITC4A$ $ITC4A$ $ITC7A$ Apatosen $CT5$ Heparin C $ITC7A$ $ITC7A$ $ITC7A$ $ITC4A$ $ITC10$ $ITC7A$ Secukinumaby $ITC73$ IgC class mAbs <sup>d</sup> $ITC7A$ $ITC7A$ $ITC7A$ $ITC10$ $ITC7A$ Secukinumaby $ITC73$ IgC class mAbs <sup>d</sup> $ITC7A$ $ITC7A$ $ITC10$ $ITC7A$ Secukinumaby $ITC73$ IgC class mAbs <sup>d</sup> $ITC7A$ $ITC10$ $ITC10$ $ITC7A$ Secukinumaby $ITC7A$ IgC class mAbs <sup>d</sup> $ITC7A$ <	CD-risk gene	CCL20	Chemoattractant for various immune cells	CTSW	Regulation of T-cell cytolytic activity	PLCG2	Transmembrane signaling of immune system receptors
IFVG $Cytokine. involved in adaptive-innatePKCBApoptosis regulationSOCS1Cytokine. regulationIRF4Regulation of mucosal Th17 cellPTPRC7-cell antigen receptor signalingSOCS1Cytokine. regulationIRF4Regulation of mucosal Th17 cellPTPRC7-cell antigen receptor signalingSOCS1Cytokine. regulationIRF4Regulation of mucosal Th17 cellPTPRC7-cell antigen receptor signalingSOCS1Cytokine. regulationMAP3K8Thelper cell differentiation and IFN7-cell antigen receptor signalingSOCS1Cytokine. regulationMAP3K8Thelper cell differentiation and IFN8PRS4SIPK5OzanimodoOCS1SOCS1Cytokine. regulationMAP3K8Tabeler cell differentiation and IFNSIPK5OzanimodoCocds2SICK9ErolizanMordiate for drugDVJBIAptorentCCL5Heparin corpoundsCCL5Heparin corpoundsCCL5Heparin corpoundsSIK1AptorentCCL5Heparin corpoundsCCL5Heparin corpoundsCCL5Heparin corporationSIK1NedocrontingSIK1NedocrontingFCR3BC-ABsbbbADRBISICH7EIO101SIK1NedocrontingFCR3BC-ABsbbADRBISICH7EIO101FCR4Abrencip (Horocronting)FCR3BC-ABsbbADRBISICH7EIO101FCR4Abrencip (Horocronting)FCR3BC-ABsbb$		DNAJB4	Heat shock protein, involved in protein folding	IdST	Adhesion and transendothelial migration	PTPN22	Negative regulator of TCR signaling, positive regulator of TLR signaling
IRF4Regulation of mucosal Th17 cell $PTPRC$ T-eell antigen receptor signaling regulation $MAP3K8$ T-heber cell differentiation gamma expressionT-heber cell differentiation and IFN gamma expression $I.17A$ Secukinumab $Known CD-drug$ $I.17A$ Secukinumab $SIPR5$ Ozanimod $CD3DE/G$ $NI-0401$ known CD-drug $I.17A$ Secukinumab $SIPR5$ Ozanimod $CD3DE/G$ $NI-0401$ target $I.17A$ Secukinumab $SIPR5$ Ozanimod $CD3DE/G$ $NI-0401$ target $I.17A$ Secukinumab $SIPR5$ Ozanimod $CD3DE/G$ $NI-0401$ target $I.17A$ Secukinumab $SIPR5$ $I.17A$ $I.0401$ $I.0401$ target $I.17A$ Secukinumab $SIPR5$ $I.0400$ $I.0401$ $I.0401$ target $I.17A$ Secukinumab $SIPR5$ $I.0400$ $I.0401$ $I.0401$ target $I.17A$ Aptorsen $I.17A$ $I.07A$ $I.0401$ $I.0401$ target $I.17A$ Aptorsen $I.17A$ $I.0401$ $I.0401$ $I.0401$ target $I.17A$ Aptorsen $I.07A$ $I.0401$ $I.0401$ $I.0401$ target $I.07A$ $I.0101$ $I.0101$ $I.0101$ $I.0101$ $I.0101$ target $I.0101$ $I.0101$ $I.0101$ $I.0101$ $I.0101$ $I.0101$ target $I.0101$ $I.0101$ $I.0101$ $I.0101$ $I.0101$ $I.0101$ target $I.0101$ $I.0101$ <		IFNG	Cytokine, involved in adaptive-innate immunity	PRKCB	Apoptosis regulation	SOCSI	Cytokine-inducible negative regulation of cytokine signaling
$MAP3K8$ Thelper cell differentiation and IFN gamma expressionNHOPOKnown CD-drug $IL17A$ SecukinumabS1PR5Ozanimod $CD3D:E/G$ NI-0401known CD-drug $IL17A$ Secukinumab $S1PR5$ Ozanimod $CD3D:E/G$ NI-0401known CD-drug $IL17A$ Secukinumab $S1PR5$ $IL17A$ $CD3D:E/G$ NI-0401known CD-drug $IL17A$ Secukinumab $S1PR5$ $IL17A$ $CD3D:E/G$ $IL10A$ Candidate for drug $DNAIB1$ Aptorsen $CCL5$ Heparin compounds $CCL5$ Heparin corpounds $CCL5$ Heparin corpoundsCandidate for drug $DNAIB1$ Aptorsen $CCL5$ Heparin corpounds $CCL5$ Heparin corpounds $CCL5$ Heparin corpoundsSIK1DabrafanibFGR $FGR3$ $IgG class mAbs^b$ $ADRB1$ $STD-I01-$ HSP90AA1Nedocronil $FGR3$ $IgG class mAbs^b$ $AL0X5AP$ FiboflapoPTGER4Rivenpost; timaprost; dinoproston $ITGB2$ $IfftegrastSLAMF7ElotuzumPDE4DAprenilastADRB2S_2-adrenergic receptor blockersADM4ALN56$		IRF4	Regulation of mucosal Th17 cell differentiation	PTPRC	T-cell antigen receptor signaling regulation		
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Candidate for drug $DNAJBI$ Apatorsen $CCL5$ Heparin compounds $CCL5$ Heparin crepositioning $SIKI$ Dabrafanib $FGR$ Dasatinib $ADRBI$ $STD-101$ $SIKI$ Dabrafanib $FGR$ Dasatinib $ADRBI$ $STD-101$ $HSP90AAI$ Nedocromil $FCGR3A$ $IgC class mAbs^b$ $ALOX5AP$ Fiboflapo $PTGER4$ Rivenprost; linaprost; dinoproston $ITGB2$ Liftegrast $SLAMF7$ Elouzum $PDE4D$ Apremilast $ADRB2$ $b_2$ -adrenergic receptor blockers $MDM4$ $ALRV-69$				FCGR3A	IgG class mAbs <sup>a</sup>	ITGAE CCR9 TGFBR1	Etrolizumab Vercirnon Mongersen $^{c}$
SIK1DabrafanibFGRDasatinibADRB1STD-101- $HSP90AAI$ Nedocromil $FCGR3A$ IgG class mAbs $ALOX5AP$ Fiboflapo $PTGER4$ Rivenprost; limaprost; dinoproston $ITGB2$ Liftegrast $SLAMF7$ Elotuzum $PDE4D$ Apremilast $ADRB2$ $\beta_2$ -adrenergic receptor blockers $MDM4$ $ALRN-69$	Candidate for drug repositioning	DNAJBI	Apatorsen	CCL5	Heparin compounds	CCL5	Heparin compounds
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PTGER4Rivenprost; limaprost; dinoprostonITGB2LifitegrastSLAMF7Elotuzum $PDE4D$ Apremilast $ADRB2$ $\beta_2$ -adrenergic receptor blockers $MDM4$ ALRN-69		HSP90AA1	Nedocromil	FCGR3A	$\lg \mathrm{G} \operatorname{class} \mathrm{mAbs}^b$	ALOX5AP	Fiboflapon
PDE4D Apremilast $ADRB2 \beta_{2}$ -adrenergic receptor blockers $MDM4$ ALRN-69		PTGER4	Rivenprost; limaprost; dinoproston	ITGB2	Lifitegrast	SLAMF7	Elotuzumab
•		PDE4D	Apremilast	ADRB2	$oldsymbol{eta}_{2}$ -adrenergic receptor blockers	MDM4	ALRN-6924

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NOTE. Heal mucosal Th17 cells, peripheral blood CTLs, and ileal mucosal CTLs show the largest number of significantly overexpressed CD-risk genes. The top 5 most significantly overexpressed CD-risk treatment of CD in humans. "Candidate for drug repositioning" refers to genes encoding targets for drugs currently approved for or under investigation in humans for other diseases than CD (Drug-Target Selection of CD-risk genes is explained in the CD-Risk Genes section in the supplement. "Known CD-drug target" refers to genes encoding targets for drugs currently approved or under investigation for genes are listed in the first section of this table. In the second section, the top 5 most significantly overexpressed known CD drug targets and candidates for drug repositioning, per cell type, are listed. Genes section in supplement).

IFN, interferon; IgG, immunoglobulin G; mAbs, monoclonal antibodies; TCR, T-cell receptor; TLR, toll-like receptor.

 $^{a}$ For example, adalimumab, etanercept, and natalizumab.

bFor example, alefacept and alemtuzumab.

 $c_{\mathrm{Indirect\ target.}}$