CHAPTER 6

Knockdown of the long non-coding RNA RP11-291B21.2 interferes with the activation of CD8+ T cells

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In progress
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**Abstract**

CD8$^+$ T cells, also called cytotoxic T cells (CTLs), are crucial effector cells in anti-viral and anti-cancer immunity. However, it remains largely unknown how the CTL compartment is shaped towards subpopulations with distinct roles even though deregulation of particular CTL subtypes may contribute to autoimmune disorders such as celiac disease, type 1 diabetes and psoriasis. Because of their cell-type-specific function as regulators of gene expression, cell activation and differentiation, long non-coding RNAs (lncRNAs) are important regulatory transcripts in immune cell regulation. We selected the lncRNA, \textit{RP11-291B21.2}, located in the vicinity of the natural killer receptor genes for further investigation because it is highly expressed in naïve CD8$^+$ T cells and downregulated in response to T cell receptor activation.

We characterized the pattern of \textit{RP11-291B21.2} expression across different T cell subsets in blood and investigated its role in intraepithelial CTL (IE-CTL) activation using single-cell RNA-seq data, co-expression network analysis and knockdown experiments. We find that \textit{RP11-291B21.2} is specifically expressed in naïve CD8$^+$ T cells and that its expression is reduced upon physiological- or in vitro-induced activation. Knockdown of \textit{RP11-291B21.2} in IE-CTLs impaired the production of proinflammatory mediators such as tumor necrosis factor alpha and interferon gamma. Together these findings indicate that \textit{RP11-291B21.2} has a dual role: it maintains the naïve/resting status of CD8$^+$ T cells and modulates the activation of effector memory T cells, antigen-experienced cells essential for mounting a rapid and strong immune defense.

**Key words:** IncRNAs, \textit{RP11-291B21.2}, CD8$^+$ T cells, NK receptors, cell activation
**Introduction**

CD8+ T cells, also known as cytotoxic T cells (CTLs), are essential in the control of infections and anti-tumor immunity. The CTL response includes a number of mechanisms, such as the release of perforins, granzymes, Fas/FasL and proinflammatory cytokines (e.g. IFNγ, TNFα), that result in the lysis of infected or transformed cells and the enhancement of effector properties of other immune cells such as monocytes, natural killer cells (NK), neutrophils and other CTLs1,2. Abnormal CTL differentiation and function is known to contribute to an attenuated immune response or to autoimmunity2.

Extensive work over recent years has shown that CTL differentiation depends on cytokines, transcription factors, DNA methylation status, histone modifications, microRNA expression and transcriptional regulation2,3. Recently, long non-coding RNAs (lncRNAs) have garnered attention due to their potential cell-type-specific and context-specific involvement in the regulation of gene expression4,5. LncRNAs have been identified as regulators of hematopoietic cell differentiation and have been shown to be differentially expressed during the activation of B cells, dendritic cells and T cells (amongst other cell types)4. Systematic assessment of the transcriptional changes in the human or mouse CD8+ T cells activated upon infection or vaccination has shown that particular lncRNAs are dynamically and specifically expressed in different subpopulations of CD8+ T cells (naïve, effector and memory), a finding that both corroborates previous findings supporting the cell-type-specificity of lncRNA expression and shows they have a role in CTL fate decisions and activation6,7.

CTLs have been recognized as key drivers in the development of tissue-specific autoimmune diseases such as type 1 diabetes (T1D), psoriasis, and celiac disease (CeD). For instance, a hallmark of CeD is extensive infiltration of intraepithelial CTLs (IE-CTLs) into the intestinal mucosa, where these IE-CTLs become abnormally activated and kill intestinal epithelial cells independently of T cell receptor (TCR) specificity, which leads to the villous atrophy characteristic of CeD8,9. An increase in the expression of the non-classical HLA-I molecules (e.g. MICA) that are recognized by NK receptors expressed on the surface of IE-CTLs (e.g. NKG2D) is specifically associated with CeD pathology10. Moreover, an upregulation of pro-inflammatory cytokines such as IL-15 contributes to the expression of NK receptors in IE-CTLs and provides co-stimulatory signals that lead to epithelial cell destruction in CeD8,11. A more thorough understanding of the mechanisms controlling CTL differentiation and activation could thus have significant therapeutic implications for CeD as well as other immune-mediated diseases.

Here we aimed to study the biological properties of a non-characterized lncRNA in CD8+ T cells. To do so we generated short-term CD8+ TCR αβ cell lines derived from the intestinal intraepithelial compartment and studied the effects of knocking down the lncRNA RP11-291B21.2. This lncRNA was selected based on its unusually high level of expression and
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CD8+ T cell expression-specificity and on in silico predictions suggesting it is involved in cell activation. Our results indicate a dual role for RP11-291B21.2 where it both maintains the naïve status of CD8+ T cells and modulates the response to stimulation once the cells have become functionally active.

Methods

Intraepithelial CTL (IE-CTL) cultures
Human biopsies were taken from CeD patients with their informed consent. All the protocols were approved by the University of Chicago Institutional Review Board. CeD individuals were defined on the basis of the presence of HLA-DQ8 or DQ2 and clinical (symptoms, response to a gluten free diet), histological (partial or total villous atrophy, crypt hyperplasia and increased IE-CTLs on duodenal biopsy) and serological (elevated anti-TG2 antibodies in blood) criteria. CD8+ TCRαβ+ short-term IE-CTL cell lines derived from duodenal biopsies from CeD individuals (n=3) were isolated and cultured as described previously12,13. In brief, cells from the intraepithelial lymphocyte compartment were isolated by mechanical disruption in RPMI (GIBCO) media supplemented with 1% dialyzed fetal bovine serum (Biowest), 1.5mM MgCl2 (Thermo Fisher Scientific) and 2mM EDTA (Corning). For IE-CTL cultures, a maximum of 10,000 cells expressing TCRαβ+ (IP26, BioLegend) and CD8α+ (RPAT-8, BioLegend) were collected via fluorescence-activated cell sorting (FACS) on a FACSAria II cell sorter (BD Biosciences). For ex vivo assessment of gene expression, a fraction of the cells (5x10³) were suspended in the lysis buffer included in the RNeasy plus micro kit (Qiagen), and these cells were subsequently analyzed by qPCR. The cells were expanded in vitro with a mix of feeder cells (irradiated heterologous peripheral blood mononuclear cells (PBMCs)) from two donors and Epstein Barr virus transformed B cells (EBV) in RPMI 1640 (GIBCO) medium with 1μg/ml PHA-L (Calbiochem), 10% human serum albumin (Atlanta Biologicals) and 100 units/ml IL-2 (NIH). The medium (RPMI supplemented with human albumin serum and IL-2) was refreshed every 2-3 days. After 12-14 days of expansion, aliquots of cells were frozen to establish a full set of cell lines for further use or re-expanded if required.

Blood-derived T cell isolation and culture
Heparinized blood samples were obtained from healthy donors. PBMCs were isolated by Ficoll density gradient centrifugation and subsequently stained with anti-CD45 (HI30, BD Biosciences), anti-CD3 (UCHT1, BD Biosciences), anti-CD4 (SK3, BD Biosciences), anti-CD8a (RPA-T8, BD Biosciences), anti-TCRαβ (IP26, BD Biosciences), anti-CD45RA (HI100, Biolegend) and anti-CCR7 (G043H7, Biolegend) antibodies. Isolation of CD4+ T cells or CD8+ T cells was performed on a FACSAria II cell sorter (BD Biosciences). For all data, an initial gate for live cells based on forward and side scatter (FSC and SSC) parameters was used. In some experiments CD8+ T cells were further separated, based on the expression pattern of CD45RA and CCR7, into naïve (CD45RA+CCR7+), effector memory (TEM, CD45RA/CCR7-), central memory (TCM, CD45RA/CCR7+)

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Population were suspended in the lysis buffer included in the RNeasy plus micro kit (Qiagen) for RNA isolation, and then qPCR was performed. For cell-line expansion, a fraction of each sorted population (5x10^3 cells) was collected in culture medium and then cultured with feeder cells as described above (see **IE-CTL cultures**).

**Transfection of siRNA**
IE-CTLs (CD8+ TCRαβ+) were thawed and re-expanded for 12 days as described above (see **IE-CTL cultures**). To estimate cell viability (>80%), cells were analyzed with LIVE/DEAD fixable Aqua Cell Stain Kit (Life Technologies) and cell activation (<10%) was determined by FSC and SSC analysis using the LSR Fortessa™ (BD Biosciences) flow cytometer. Cells were suspended at a density of 1x10^6 cells/ml in Accell siRNA delivery medium (Dharmacon), supplemented with 100 units/ml IL-2 (NIH) and transfected with Accell siRNA duplexes designed for the non-targeting control (Dharmacon) or RP11-291B21.2 (Dharmacon) at a final concentration of 1μM and plated in 96-well plates at a density of 2x10^5 cells/well. The conditions for siRNA treatment were previously determined (data not shown). Untreated cells were included as negative control (wild type (WT)). After 24 hours, the medium was refreshed, the transfection was repeated and the cells were incubated for additional 48 hours.

**IE-CTL stimulation**
After siRNA treatment (72 hours), cells were washed and suspended at a density of 1x10^6 cells/ml in RPMI medium 1640 (GIBCO) containing 10% human serum albumin (Atlanta Biologicals) and 100 units/ml IL-2 (NIH). Cells were either left unstimulated or stimulated with 1 μg/ml of plate-bound anti-CD3 antibody (eBioscience). This treatment emulates the stimulation provided by the binding of antigen-presenting cells to the TCR. A fraction of the cells was treated with 1 μl/ml of Golgi stop and Golgi plug to inhibit protein release (both from BD Biosciences) and harvested for flow cytometry. After 3 hours, cells were centrifuged for 10 minutes at 400g, and the supernatants were collected and stored at -80°C until further protein detection. The pellets were suspended in the lysis buffer included in the mirVana™ miRNA isolation kit (Invitrogen) for RNA isolation and downstream procedures including qPCR and RNA-seq.

**Flow cytometry**
After stimulation, cells were collected and washed with PBS and then stained with LIVE/DEAD fixable Aqua Cell Stain Kit (Life technologies), anti-CD3 APC-Cy7 (UCHT1, Biolegend) and anti-CD8α BV650 (RPA-T8BD, Biosciences). Subsequently, cells were washed and fixed in cytofix/cytoperm buffer (BD Biosciences), washed with 400 μl of Perm/wash buffer (BD Biosciences), and then incubated with conjugated antibodies to IFNγ APC (4SB3, Biolegend), TNFα PECy7 (Mab11, Biolegend) and CD107α BUV396 (H4A3, Biolegend) for 1 hour at 4°C. The cells were washed and analyzed on the BD LSR Fortessa™ (BD Biosciences) flow cytometer. Data was analyzed with FlowJo (Treestarts). Results are expressed as the frequency of cytokine-expressing cells in the gated CD3+ CD8α+ population.
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RNA sequencing (RNA-seq)
Total RNA from unstimulated or stimulated IE-CTLs cell lines was isolated with the mirVana™ miRNA isolation kit (Invitrogen). RNA concentration and quality were assessed on the Nanodrop 1000 Spectrophotometer (Thermo Scientific) and the high-sensitivity RNA analysis kit (Experion, software version 3.0, Bio-Rad). RNA-seq libraries were prepared using the Quant seq 3’ kit (Lexogen) according to the manufacturer’s instructions. Sequencing was performed on the Nextseq500 (Illumina), yielding at least fifteen million sequence reads per sample. The fastQ files were trimmed for low quality reads, adaptors and poly-A tails. Trimmed fastQ files were aligned to build a human_g1k_v37 ensemble Release 75 reference genome using hisat15 with default settings and sorted using SAMtools16. To perform gene-level quantification, we used HTSeq-count17 using default mode=union. A modified Ensembl version 75 gtf file mapping to the last 5’ 500 base pairs (bps) per gene was used to prevent counting of reads mapping to intra-genic A-repeats during gene annotation. Data normalization of raw counts (variance stabilizing transformation (VST)) and differential expression analysis between conditions were performed using the DESeq218 package in R. Differentially expressed genes (DEGs) were defined based on a False Discovery Rate (FDR) ≤0.05 among treatments. Principal component analysis (PCA) was performed using VST counts for all the samples. R Base functions (v3.4) including PCA, Venn diagrams and heatmap were used to display overall or specific transcriptional profiles. Gene-set enrichment analysis19 and Reactome pathways20 were used to identify biological processes and pathways enriched in different sets of DEGs.

For the RNA-seq dataset described in Fig. 1 (unpublished data, Mayassi T, et al), short-term TCRγδ (VD1) and CD8+ TCRαβ CD8+ (CD8) blood- or intestinal-derived T cell lines were expanded for 12 days, and then left untreated (unstimulated) or treated with 1.5 μg/ml of anti-CD3 or anti-TCRγδ, respectively, for 4 hours. RNA was isolated with the mirVana™ miRNA isolation kit (Invitrogen) and used for bulk RNA-seq (Illumina TruSeq Ribo-Zero) and sequenced on a Hiseq 2500 instrument (Illumina) using default parameters (single end, 1x50bp). A standard pipeline was used to filter the sequencing reads. Reads were mapped to the build 37 human reference genome using hisat15. Read counts were normalized to reads per kilobase of transcript, per million mapped reads (RPKM). Differential expression analysis was performed using the DESeq2 package18. Genes were considered differentially expressed based on an FDR ≤ 0.05 among treatments.

RT-qPCR
Total RNA from cell lysates was isolated using the mirVana™ miRNA isolation kit (AMBI-ON, catalog AM1561) for high numbers >5000 cells or the RNeasy plus micro kit (Qiagen) for low cell numbers <5000 cells. cDNA was prepared using either RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) for inputs >5000 cells, or SuperScript VILO cDNA synthesis kit (Invitrogen) for inputs <5000 cells. qPCR was performed using the Syber green mix (Bio-Rad) and run in a Quant studio 7 flex real time system (Applied Biosystems). The primers used were: RP11-219B21.2 5’ ACCAGTAACAGGCATTGGGA, RP11-291B21.2
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3′GCAAGTTGTCCACACGGTAC, GAPDH 5′ ATGGGGAAAGTGAGGCG, GAPDH 3′ GGGTCTATTGATGGCAACATA. Expression of GAPDH was included as internal control to calculate the relative gene expression using the $2^{-\Delta\Delta CT}$ method. The values of relative expression were converted to percentages by setting the WT cells or CD8+ naïve as reference (100% expression).

**Olink**

Levels of 92 pro- and anti-inflammatory proteins in supernatants were measured by the Immuno-Oncology panel 1 (Olink Bioscience) as described previously. In brief, antibodies coupled with specific oligonucleotide probes bind to their specific target proteins, and the sequence is then quantified by RT-qPCR amplification. The results are given as the normalized protein expression (NPX) value on log-2 scale. The limit of detection (LOD) for each analyte was defined as three standard deviations above the background, and proteins with levels lower than LOD were excluded from the analysis. Net or total protein production in response to anti-CD3 stimulation was calculated by subtracting the level of protein production in unstimulated cells from that in the corresponding stimulated sample. Supplementary Fig. 1 provides a summary of the methods and experimental approaches used to profile the expression of RP11-291B21.2 in different immune cells and its potential function in CD8+ T cells.

**Statistical analysis**

The Shapiro-Wilk normality test was performed in R to assess the distributions of the flow cytometry, qPCR and Olink data. Differences within two groups were tested using unpaired one-tailed student’s t-test. Results were plotted using GraphPad Prism (GraphPad software) and presented as individual measurements (line plots) or mean ± standard error of the mean (SEM) (bar plots) from a representative experiment. Analysis of biological pathways and processes associated to individual genes or a group of genes were carried out using Gene Network v2.0 (www.genenetwork.nl) and Reactome pathways. To compare co-expression patterns between RP11-291B21.2 and neighboring genes (cis genes in a 1 mega base (Mb) window centered on the location of the lncRNA) or distant genes (trans genes, outside the 1Mb window), we calculated Spearman correlation coefficients. Heatmaps were made using the R base function “pheatmap” to depict relevant patterns of gene expression or normalized protein secretion across the treatments. A brief description of the statistical tests and significance are described in each figure legend if applicable.

**Results**

LncRNA RP11-291B21.2 is co-expressed with NK receptor genes in T cells

To characterize the transcriptional signatures of intestinal lymphocytes (IELs) and their counterparts in blood, RNA-seq was performed in short-term TCRγδ (VD1) and CD8+ TCRαβ (CD8) blood- or intestinal-derived cell lines (unpublished data, Mayassi T, et al; for more details see Fig. 1 and RNA-seq section in Methods). Among the genes differentially expressed between
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In cell subsets, we identified one IncRNA, RP11-291B21.2, with exceptionally high expression (~40 RPKM; Fig. 1A) compared to the mean expression of all other IncRNAs (0.33 RPKM on average; Fig. 1A). This level of expression is similar to the mean expression level of protein coding genes (38 RPKM; Fig. 1A), which is surprising given that IncRNA expression is generally lower than that of coding mRNAs. Expression of RP11-291B21.2 was higher in cells derived from intestinal tissues compared to blood-derived VD1 cells. Additionally, a reduction in the expression of RP11-291B21.2 was noted after TCR stimulation, irrespective of the source (blood or intestine). Taken together, the high constitutive level of RP11-291B21.2, its tissue-specific expression profile and its modulation in response to stimulation suggest it has a role in the normal physiology of CTLs but also plays a role in the activation process of different T cell populations.

The biological role of the vast majority of IncRNAs, including RP11-291B21.2, is unexplored. However, examining their genomic co-localization with protein-coding genes and their tissue-specific expression profiles and performing co-expression analysis could provide some clues about their function. To assess whether RP11-291B21.2 is co-expressed with genes in particular pathways, we performed Spearman correlation analysis on the transcriptomes obtained from TCRγδ (VD1) and CD8+ TCRαβ (CD8) blood- or intestinal-derived cell lines and plotted the correlation of all genes in a 1Mb window centered on RP11-291B21.2 (cis) and the top 20 correlated trans genes with RP11-291B21.2 (>5Mb away). Strikingly, genes in cis were more strongly correlated than trans genes, suggesting that RP11-291B21.2 potentially has a local cis-regulatory role. Among the genes strongly correlated with RP11-291B21.2 in cis are genes encoding for activating (e.g. KLRC2, KLRK1, KLRC3, KLRC4-KLRK1) and inhibitory (KLRC1) NK receptors; KLRD1, which encodes the NK receptor adapter molecule CD94; and STYK1, a recently described hallmark gene for NK cells that may act as a regulator of the PI3K/AKT/mTOR pathways (Fig. 1B). NK receptors are mainly expressed by CTLs and NK cells and are known to recognize virus-infected cells, tumorigenic cells or cells expressing abnormal levels of MHC-I ligands. These receptors are also involved in regulation of TCR stimulation and CTL effector functions. In trans, genes like MN1 and TEAD3 (encoding for transcriptional regulators) and several IncRNAs and microRNAs (e.g. miR-641, RP11-543E8.2) with poorly characterized or unknown functions were correlated most strongly (Fig. 1B). Although the overall correlation was similar in all conditions (unstimulated or stimulated cells, blood- or intestinal-derived cells), we found small differences in the correlation of certain genes in particular cell types. For instance, immune genes (CLEC7A) and taste receptor genes (e.g. TAS2R50, TAS2R13) were particularly highly correlated with RP11-291B21.2 expression in CD8+ IELs. In fact, the different cell types could be clustered based on the pattern of correlation (e.g. a group of VD1 cell vs. CD8+ T cells; or a group of unstimulated VD1 vs. stimulated VD1). This suggests that the co-expression between RP11-291B21.2 and NK genes in CTLs is strong regardless of the cellular or stimulatory context, whereas the co-expression with the ‘non-NK cell genes’ is more cell-type- and stimulation-specific.
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To delineate the biological processes in which RP11-291B21.2 might operate, we conducted pathway analysis using Reactome (http://www.reactome.org) on the 36 most correlated genes (correlation coefficient >0.4) in the TCRγδ VD1 IEL cells where RP11-291B21.2 is most highly expressed. The correlated genes were significantly enriched in biological processes such as ‘DAP12 signaling’ and interaction between lymphoid and non-lymphoid tissues biological processes (Fig. 1C). Interestingly, DAP12 is a key tyrosine kinase binding protein (TYROBP) that acts as an activating signal transduction element in NK receptors.

Co-expression of genes in cis may be a consequence of co-localization within a single topological domain, or be based on the shared function and/or regulation of genes, or both. To assess this, we plotted the 1Mb genomic region of RP11-291B21.2 and colored the cis genes according to their correlation coefficient (Fig. 2). Among the genes in close proximity to each other and linked to the same biological function (e.g. NK receptors versus taste receptor genes) we found similar degrees of correlation (e.g. KLRC2, KLRC3 and KLRC4 (high) vs. TAS2R9 and TAS2R10 (low)). Furthermore, we also noted that some genes in close proximity to RP11-291B21.2 correlate less strongly (e.g. EIF2S3L) than cis genes located further away (e.g. KLRK1). Thus, we propose that co-expression of genes in cis with RP11-291B21.2 is mostly based on shared function and not just a consequence of proximity, which could mean that RP11-291B21.2 plays a regulatory role in NK receptor transcription or function. We also observed several lncRNAs within a distance of ~100kb (on the same chromosome) from RP11-291B21.2 with significant/suggestive correlative strength. Co-expression analysis performed in GeneNetwork predicted a function for two of the lncRNA genes (RP11-277P12.20 and RP11-277P12.9) most correlated with RP11-291B21.2 in NK cell cytotoxicity and antigen presentation (data not shown). This suggests that other lncRNAs may work together with RP11-291B21.2 in cis to regulate genes with a shared role in NK cytotoxicity.

In addition, we interpreted DNase I hypersensitive data from publicly available data generated from T cells as an indicator of gene regulatory regions such as promoters and enhancers. Interestingly, the genes with higher co-expression with the lncRNA (correlation coefficient >0.4) were found to co-localize within DNase I regions (mostly at genes located at positions chr12: 10,350,000-10,900,000) specific for CD8+ T cells and NK cells (Fig. 2). This overlap was most notable in the loci containing the NK receptor genes. Furthermore, we found a dense region of DNase I peaks overlapping with RP11-291B21.2 in CD8+ T cells (marked with a red star), indicating the presence of a regulatory region or active transcription occurring at the transcription start site of the lncRNA, and this is specific for CTLs (Fig. 2).

Other data sources, including the human catalog of lncRNAs and GeneNetwork v2.0, also suggest a role for RP11-291B21.2 in the regulation of the immune response, NK cytotoxicity and TCR signaling, amongst others (Supplementary Fig. 2). Taken together, our observations point to a role for RP11-291B21.2 in CD8+ T cell activation either in cooperation with NK receptors or through regulation of them.
Knockdown of the lncRNA SIAE with correlation coefficient >0.4 in the VD1-stimulated samples (n=7 cell lines). Size of bar denotes the significance (-log 10 qvalue) of each biological pathway.

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**Figure 2.** RP11-291B21.2 locus and cis genes. Spearman correlation analysis was performed with respect to RP11-291B21.2 (described in Figure 1). University of California Santa Cruz (UCSC) tracks were modified to show genes in a 1Mb-region centered on RP11-291B21.2 and their respective Spearman correlations. Arrows indicate location, direction (sense or antisense) and gene correlation (color chart, upper-right corner). Grey arrows correspond to genes excluded from the analysis (undetectable in our dataset). Gene names in black or grey indicate correlation >0.4 or lower, respectively. Red box shows RP11-291B21.2 genomic position. Lower track corresponds to DNase I profile in primary blood-derived CD8+ T cells taken from Epigenomic roadmap."
**RP11-291B21.2 is expressed in CD8+ T cells with a naïve expression profile**

To confirm that **RP11-291B21.2** is truly specific to CD8+ T cells, we assessed its expression profile in cells freshly isolated from blood. We used available RNA-seq data from seven major immune cell types purified by cell sorting\(^3^2\) and single-cell RNA-seq (scRNA-seq) data\(^3^3\) as described in Supplementary Fig. 1. The level of **RP11-291B21.2** was found to be highly expressed in CD8+ T cells, lowly expressed in memory T cells and NK cells, and non-detectable in B cells, CD4+ T cells, granulocytes and monocytes (Fig. 3A). Moreover, T-distributed stochastic neighborhood embedding (t-SNE) analysis of PBMC scRNA-seq data identified 12 major clusters of cell types (Fig. 3B) based on the expression of canonical gene markers (Fig. 3C). **RP11-291B21.2** is highly expressed within a subset of CD8+ T cells (Fig. 3D, orange dots marked with a red oval). The cells that express the IncRNA at high levels (CD8+ **RP11-291B21.2**+) also express genes (e.g. CD3D, CD8A, CD8B) that define a CD8+ T cell cluster along with genes typically associated with a naïve status such as CCR7 and SELL (gene encoding for CD62L) (Fig. 3C, red boxes). Additionally, CD8+**RP11-291B21.2**- cells do not express NKG7 (a poorly characterized NK receptor), in contrast to cells that do not express the IncRNA (CD8+**RP11-291B21.2**- cells).

Analysis of genes differential expressed between CD8+**RP11-291B21.2**+ and CD8+**RP11-291B21.2**- cells revealed that the downregulated genes in CD8+**RP11-291B21.2**+ cells (e.g. KLRB1L, CD69, GZMHB, GZMB; Fig. 3A in blue) are associated with processes including cytotoxicity and immune activation (Fig. 4B, top 10 Reactome pathways in downregulated genes). Conversely, the upregulated genes (Fig. 4A in red) include well-established naïve cell markers (e.g. CCR7, SELL\(^3\)) and LEF-1 (encoding for TCF-1), a regulator of T cell quiescence\(^3^4\), amongst others. These results indicate that **RP11-291B21.2** is specific to CD8+ T cells that display a naïve or resting expression profile.

**RP11-291B21.2 expression decreases upon functional activation of CD8+ T cells**

To further characterize **RP11-291B21.2**+ cells, we evaluated the expression of this IncRNA in different blood-derived CD8+ T cells sorted according to the surface markers CD45RA and CCR7 into naïve, TCM, TEMRA and TEM cells (Fig. 5A shows the gating strategy), with each having different functional properties\(^3\). We also included CD4+ T cells from blood as negative controls and IELs (TCR\(\gamma\delta\) and CD8+ TCR\(\alpha\beta\)) to determine whether these cells express **RP11-291B21.2**+ **ex vivo**. As predicted, based on the scRNA-seq profiles and the analyses described above (Fig. 3-4), **RP11-291B21.2** was expressed in all the sorted cell types except CD4+ T cells (Fig. 5B). Here we observed that naive CD8+ T cells exhibit the highest levels of the IncRNA, followed by TCM, TEMRA and TEM, respectively (Fig. 4B, left), indicating that the level of **RP11-291B21.2** decreases with rising functional activation status. Additionally, biopsy-derived T cells exhibited levels of **RP11-291B21.2** comparable to those in TCM cells in blood, which confirms that the expression of **RP11-291B21.2** in intestinal CD8+ and VD1 cell lines (described in Fig. 1A) is not due to artifacts generated during **in vitro** expansion.
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Figure 3. RP11-291B21.2 is expressed in CD8+ T cells with a naïve expression profile. (A) Expression level of RP11-291B21.2 (RPKM) in different immune cells (sorted populations followed by bulk RNAseq, representative of two experiments). (B-D) scRNA-seq data previously generated from PBMC. (B) t-SNE plot displaying the main immune-cell types in PBMC identified with canonical markers and labeled with different colors. (C) Violin plots showing the expression distribution of representative marker genes across the cell clusters. (D) Expression pattern of RP11-291B21.2 in the different cell clusters inferred in the full scRNA dataset. Colors yellow to red indicate RP11-291B21.2 expression (low to high). Red ovals and rectangles mark the population where this lncRNA is highly expressed.
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Figure 4. Downregulated genes in CD8+ RP11-291B21.2+ T cells are associated with cell cytotoxicity and activation. (A) Top 40 DEGs between CD8+ T cells expressing (CD8+ RP11-291B21.2+) or not expressing (CD8+ RP11-291B21.2-) RP11-291B21.2. Downregulated (blue) or upregulated (red) genes in CD8+ RP11-291B21.2+ cells vs. CD8+ RP11-291B21.2- cells (color key, log 2-fold change). (B) Biological pathways (Reactome and KEGG) overrepresented in the set of differentially genes described in (A). Bar length indicates significance (-log 10 p-value).
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To determine if the level of RP11-291B21.2 is reduced with the activation of the CD8+ T cells and remains low or undetectable in CD4+ T cells, we expanded the sorted populations by co-culturing them with a mix of allogenic PBMCs and EBV cells (Fig. 5B center and right panels). The allogenic co-culture system is a well-known strategy to promote extensive proliferation that drives the cells towards a more functionally activated status, for example by inducing cytokine production. After co-culture, RP11-291B21.2 expression was downregulated in the different populations of CD8+ T cells (Fig. 5B, central panel). Moreover, the IncRNA level was reduced even more after a second co-culture in CD8+ T cells (Fig. 5B, right panel), but was unaffected in CD4+ T cells. Overall, our observations indicate that RP11-291B21.2 is reduced when CD8+ T cells become more activated or gain an "effector status", suggesting that the IncRNA actively maintains a naïve status when the IncRNA is expressed at high levels or represses the mechanism that allows cells to differentiate towards effector or memory T cell (Fig. 5C).

**RP11-291B21.2 knockdown impairs the CD8+ T cell response upon TCR activation**

To further explore the functional role of RP11-291B21.2 in CD8+ T cells, we knocked down the IncRNA in CD8+ TCRαβ IEL (IE-CTLs) cell lines from intestinal tissue using siRNAs. IE-CTLs cell lines were used because the optimization and execution of knockdown experiments requires a large number of cells and because IE-CTLs can be expanded to the required numbers while exhibiting considerable levels of the IncRNA (as shown in Fig. 1). IE-CTLs have “activated, yet resting” properties, inherent to tissue-resident effector memory T cells, making them a suitable model to study RP11-291B21.2. After siRNA treatment, we measured classical markers of activation and degranulation of CTLs (IFNγ, TNFα and CD107α) using intracellular FACS. Here we found that the expression of these markers was low under resting conditions, irrespective of the treatment (Fig. 6A and data not shown; <0.3% in WT, scrambled non-targeting siRNA (SCR) or knockdown (KD) unstimulated cells), but increased 8- to 15-fold after anti-CD3 treatment in all biological replicates compared to their respective unstimulated sample. Moreover, we found a reduced percentage of cells responding to anti-CD3 stimulation (IFNγ+, TNFα+ and CD107α+) in SCR and KD cells compared to WT cell lines (Fig. 6B). This effect, while not statistically significant, was stronger in the KD cells, indicating that RP11-291B21.2 may interfere with the response to TCR stimulation in IE-CTLs and supporting our hypothesis that this IncRNA could contribute to the control of CD8+ T cell activation.

**RP11-291B21.2 knockdown reduces the expression of pro-inflammatory genes responding to TCR stimulation**

To further characterize the effects of reduced RP11-291B21.2 expression on cell activation, we assessed the major transcriptional changes upon knockdown using RNA-seq. We confirmed the knockdown of the IncRNA to ~40% of WT expression levels in RNA-seq data (Supplementary Fig. 3) and in qPCR data (data not shown). We also found that the non-targeting siRNA (SCR) affected the level of RP11-291B21.2. However, this effect was minor compared with the targeting siRNA (on average ~13% SCR vs. ~40% in KD cell lines).
Knockdown of the lncRNA RP11-291B21.2 interferes with the activation of CD8+ T cells. The main surface markers of each cell population are shown (CD45RA, CCR7, CD62L, CD45RO, CD69, CD103). The mean expression across functional CD8+ T cell populations decreases with the functional activation status of CD8+ T cells.

Figure 5. Expression of RP11-291B21.2 after the first (center) or second allogenic stimulation (right). Data is presented as percentage of CD8+ T cells. Freshly isolated PBMC-derived or intestinal biopsy-derived cells were stained with specific antibodies and sorted (A, B) and CCR7 (C). Ex-vivo activated PBMC-derived or intestinal biopsy-derived cells were further separated based on the expression of CD45RA or CCR7 (dot plot, showing the gating strategy (upper panel)). (A) Blood-derived CD8+ T cells were further separated based on the expression of CD45RA or CCR7 (dot plot, showing the gating strategy (upper panel)). (B) PBMC-derived or intestinal biopsy-derived cells were stained with specific antibodies and sorted (A, B (left panel)). (A, B) Expression profiling of RP11-291B21.2 in different cell types: Blood-derived CD8+ T cells, kidney-derived CD8+ T cells, or intestinal biopsy-derived CD8+ T cells. (C) Schematic representation of CCR7+ TEMRA and CD4+ T cells or intestinal-derived T cells (CD8+ TCR).
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Figure 6. RP11-291B21.2 knockdown impairs CD8+ T cell response to TCR activation. CD8+ TCRαβ+ short-term IE-CTL cell lines derived from duodenal biopsies (n=3) were left untreated (WT) or transfected with non-targeting siRNA (SCR) or RP11-291B21.2 siRNA (KD) for 48 hours, then stimulated with 1 µg/ml of anti-CD3. Unstimulated cells were included as control. The expression of CD107α, IFNγ and TNFα was quantified by intracellular flow cytometry in the gated CD3+ CD8α+ population. (A) dot plots (n=1 representative cell line) or (B) line plots (n=3 cell lines) are shown. Numbers indicate the percentage positive cells. One-sided t-test was used to assess differences between groups. No statistical differences were found.
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We performed PCA on the normalized read counts across all the samples and treatments to study the overall distribution of the transcriptome. The first two principal components explain the majority of the variance (85%, Fig. 7). Two major clusters (grouped with circles) corresponding to the unstimulated and anti-CD3 stimulated samples were found, thus we observed strong effects on gene expression as a consequence of anti-CD3 stimulation.

Next, we evaluated the transcriptomic response to anti-CD3 stimulation. In brief, we compared the gene expression between unstimulated WT, SCR or KD samples and their corresponding anti-CD3 stimulated samples to determine DEGs (Fig. 8A), and then identified unique or shared DEGs in response to anti-CD3, which are illustrated in a Venn diagram (Fig. 8B). In total, 149 DEGs were identified, 88 of them responding uniquely in one condition (76 in WT, 7 in SCR and 5 in KD). Reactome analysis of DEGs found only in WT cells indicates enrichment in cell signaling and immune activation (e.g. RGS1, RSG12, S1PR4, GBP2, NFKB1, PTPN7, IL2RA; data not shown), whereas the unique genes responding to SCR or KD were...
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Figure 8. RP11-291B21.2 knockdown reduces the expression of pro-inflammatory genes responding to CD3 stimulation.

RNA-seq was performed on IEL-CTLs CD8+ TCRαβ+ IE-CTL cell lines (WT, SCR, KD) unstimulated or anti-CD3 stimulated. (A) General workflow of the differential expression analysis strategy. We compared the gene expression between unstimulated and respective anti-CD3 stimulated samples to identify DEGs among treatments responding to anti-CD3 stimulation. We then compared these DEGs among controls and KD cell lines (WT vs. KD, WT vs. SCR and SCR vs. KD) to identify unique or shared DEGs in response to anti-CD3, which are shown in a Venn diagram (B). The red circle marks DEGs common to all treatments (n=37). (C-D) Absolute or (D) normalized Log2 FC gene expression pattern and (E) overrepresented biological Reactome pathways for these genes. Bar size represents significance (Log 10 (q value)).
not enriched in any biological pathway. Next, we looked at the expression profile and biological role of shared DEGs (Fig. 8C, D). Among these we found genes relevant in the context of inflammation (e.g. IFNG, IL8, IRF2, RELB, FOS, TNF, JUNB) and cytotoxicity (GZMB) (Fig. 8D), as well as genes involved in biological processes such as immune cell differentiation and cytokine production (Fig. 8E). Interestingly, while these genes are differentially expressed upon anti-CD3 stimulation in all conditions, their response is least profound in KD IE-CTLs, in agreement with the results of the FACS analysis upon stimulation in Fig. 6. Surprisingly, we do not find evidence that the cis NK receptor genes strongly co-expressed with RP11-291B21.2 (Fig. 1) were differentially expressed under any of the conditions we analyzed (data not shown). This could be due to several biological factors and/or technical reasons that we discuss in detail below.

We next determined whether RP11-291B21.2 knockdown could also interfere with protein secretion, as this is a direct measure of the cytotoxicity and functionality of IE-CTLs. Here we used Olink, an array designed for simultaneous quantification of 92 proteins, to evaluate changes in secretion of targeted proteins (pro- and anti-inflammatory cytokines and chemokines) (Supplementary Fig. 4). As a quality control, proteins with >50% samples below the limit of detection were excluded (65 remained). We observed a significant increase in the production of several cytokines and chemokines in the anti-CD3 stimulated cells compared to their respective unstimulated controls (~50% of the remaining proteins; e.g. IL-6, GZMB; data not shown). In contrast, no significant differences were found in the net production of proteins between WT-, SCR- or KD-stimulated samples. Nonetheless, the secretion of proinflammatory proteins whose corresponding encoding gene was downregulated upon RP11-291B21.2 knockdown followed the same trend in the majority of the cases (e.g. IFNγ, IL-13, CCL4; Supplementary Fig. 4 and Fig. 8D), indicating that the transcriptional and functional response to TCR stimulation is impaired after IncRNA knockdown and further supporting a potential role for RP11-291B21.2 as part of the regulatory mechanism of CD8+ T cell activation.

Discussion

Although IncRNAs are known to influence cell fate and activation of immune cells in a cell-type-specific manner, their precise functions are still largely unknown. In the current study, we identified a novel IncRNA, RP11-291B21.2, that is highly expressed in blood-derived CD8+ T cells exhibiting naïve characteristics. The gene encoding RP11-291B21.2 is located in a locus encompassing several NK receptors, some of which are co-regulated with this IncRNA. By investigating the pattern of expression of the IncRNA in different functional subpopulations of freshly isolated CD8+ T cells or after induced activation/expansion in vitro, we found that RP11-291B21.2 is reduced concomitantly with cell activation. Downregulation of RP11-291B21.2 expression led to a reduced transcriptional response and impaired production of pro-inflammatory cytokines in IE-CTLs.
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Although 16,000 IncRNA have now been mapped to the human genome by the Gencode consortium (Gencode Human Version 30; https://www.gencodegenes.org), only about a hundred of these have had a function assigned to them. Co-expression analysis has been used to predict gene functions based in the principle of guilt-by-association, where genes involved in the same biological process usually present comparable expression patterns\(^{29,39}\). The strong expression correlation that we observed between NK receptor genes and RP11-291B21.2, together with in-silico predictions, point to a collaborative action between these genes in tuning the activation of CTLs. In agreement with these observations and reports showing cell-type-specificity and context-dependent expression of IncRNAs\(^4\), we found that RP11-291B21.2 is specifically expressed in CD8+ T cells, allowing discrimination between CD8+ T cells and other immune cells. Furthermore, RP11-291B21.2 level corresponds to naïve differentiation states of CD8+ T cells (Fig. 5), indicating that it may be relevant in the CD4/CD8 cell fate decision and to the activation status within the CD8+ compartment. However, we did not observe significant changes in the expression of NK receptors upon RP11-291B21.2 knockdown (data not shown). Although it is possible that this mechanism occurs under conditions more representative of the intestinal tissue environment, it is also plausible that technical factors such as the delivery medium used during the transfections (e.g. low serum medium, reaction buffers) or the effect of non-targeting siRNA on the activation of IE-CTLs may have affected the response to stimulation, precluding detection of less strongly differentially expressed genes upon knockdown (depicted in Fig. 7). Thus, alternative strategies such as the use of other non-targeting siRNAs, and perhaps even improved targeting methods like the CRISPR inhibitory system (CRISPRi) that allows titration of the targeted genes\(^{40}\), could strengthen future findings and elucidate how RP11-291B21.2 modulates CTL activation.

In our knockdown experiments, we examined the role of RP11-291B21.2 in IE-CTLs, the effector cells in CeD, in response to TCR stimulation. However, it is important to note that CeD patients exhibit an increase in the activation of NK receptors on the surface of IE-CTLs\(^{41}\) as well as the upregulation of non-classical MHC-I molecules and stress-related molecules, both of which are ligands for NK receptors and known to cause IE-CTL activation. Furthermore, CeD is characterized by upregulation of pro-inflammatory cytokines (e.g. IL-15, IFN) in the intestinal mucosa that, in turn, boost IE-CTL activation and the expression of NK receptor ligands in the intestinal epithelial cells\(^{8,11}\). Therefore, it is possible that the putative regulatory role of RP11-291B21.2 in NK receptor function may not be observed under the non-physiological stimulatory conditions that we used in this study. Further analysis under stimulation conditions more representative of in vivo conditions could provide clues about the potential role of RP11-291B21.2 in CTL activation.

LncRNAs have been described to function in both cis and trans. Some IncRNAs can regulate the expression of nearby cis genes directly through their inherent regulatory properties or via the recruitment of regulatory and epigenetic modifiers to the locus\(^{42}\). Given the overlap between genes highly co-expressed with RP11-291B21.2 and DNase I regions in cis (Fig. 2),
Knockdown of the lncRNA RP11-291B21.2 interferes with the activation of CD8+ T cells.

these seem plausible mechanisms to regulate NK gene expression. Alternatively, IncRNAs are also known to have a structural role, for instance serving as scaffolds for signaling proteins, and some IncRNAs may actually combine these features. As a consequence, IncRNAs, and perhaps RP11-291B21.2, may participate in interactions with key signaling transducers. A predominant \textit{trans} effect in IE-CTLs could be an alternative explanation for the lack of correlation between RP11-291B21.2 and NK receptor expression upon its KD in IE-CTLs. Alternatively, RP11-291B21.2 may not regulate the expression of NK receptors directly, but rather regulate the function of these receptors indirectly, for example by interacting with the signaling domains and phosphorylation processes of upstream transcription factors or downstream intracellular signaling molecules in the cytoplasm. Based on our current findings, we cannot exclude either regulatory mechanism. Further RNA localization and pulldown assays that interrogate the possible physical interactions between RP11-291B21.2 and other proteins that may act in \textit{cis or trans} are necessary to elucidate these possible functional mechanisms.

In summary, we describe a CD8+ T cell-specific IncRNA that seems to play a dual role in which it both maintains a naïve cellular status and contributes to the regulation of the proinflammatory response in CD8+ effector memory T cells. CTLs are pivotal effector cells in anti-viral and anti-cancer immunity. Dysregulation of CD8+ T cells contributes to the development of autoimmune-mediated diseases such as CeD. Unveiling the processes involved in CTL homeostasis and imbalance is important to understand the development, activation and maintenance of the CTL compartment, and will ultimately help to design therapeutic targets to modulate CTL properties. Although the precise mechanism underlying the role of RP11-291B21.2 remains to be determined, our work provides the basis for further exploration of this IncRNA in CTL physiology and in conditions of abnormal inflammation where dysbalanced CTL activation leads to tissue destruction.

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**Supplemental information**

**Profiling IncRNA across different immune cells**

Diagram summarizing the main experimental procedures conducted to profile the expression of RP11-291B21.2 in different immune cells (upper panel) and to investigate the potential function of this IncRNA by performing a knockdown experiment (lower panel).

**Supplementary Figure 1. General methods.** Diagram summarizing the main experimental procedures conducted to profile the expression of RP11-291B21.2 in different immune cells (upper panel) and to investigate the potential function of this IncRNA by performing a knockdown experiment (lower panel).

**RP11-291B21.2 knockdown**

**Supplementary Figure 2. RP11-291B21.2-predicted biological processes and pathways.** The main GO biological processes (left panel) and Kegg pathways (right panel) predicted by Gene Network v2.0 for RP11-291B21.2 are shown. Bar size indicates the significance of each pathway.
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Supplementary Figure 3. RP11-291B21.2 knockdown confirmation. The relative expression values for RP11-291B21.2 (VST, taken from RNA-seq) in the different CD8+ TCRαβ+ IE-CTLs cell lines are expressed as percentage by setting WT control cell lines as reference (100%).

Supplementary Figure 4. Reduced production of pro-inflammatory mediators upon RP11-291B21.2 knockdown. Pro- and anti-inflammatory proteins secreted by CD8+ TCRαβ+ IE-CTL cell lines (WT, SCR or KD) were quantified by Olink and expressed as arbitrary units (NPX). Heatmap shows the net cytokine production in anti-CD3 stimulated cells. Color code indicates the cytokine level (orange=high to blue=low).
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