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The 3p21.31 genetic locus promotes progression to type 1 diabetes through the CCR2/CCL2 pathway

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ABSTRACT

Multiple cross-sectional and longitudinal studies have shown that serum levels of the chemokine ligand 2 (CCL-2) are associated with type 1 diabetes (T1D), although the direction of effect differs. We assessed CCL-2 serum levels in a longitudinal cohort to clarify this association, combined with genetic data to elucidate the regulatory role of CCL-2 in T1D pathogenesis.

The Diabetes Autoimmunity Study in the Young (DAISY) followed 310 subjects with high risk of developing T1D. Of these, 42 became persistently seropositive for islet autoantibodies but did not develop T1D (non-progressors); 48 did develop T1D (progressors). CCL-2 serum levels among the three study groups were compared using linear mixed models adjusting for age, sex, HLA genotype, and family history of T1D. Summary statistics were obtained from the CCL-2 protein quantitative trait loci (pQTL) and *CCR2* expression QTL (eQTL) studies. The T1D fine mapping association data were provided by the Type 1 Diabetes Genetics Consortium (T1DGC).

Serum CCL-2 levels were significantly lower in both progressors ($p = 0.004$) and non-progressors ($p = 0.005$), compared to controls. Two SNPs (rs1799988 and rs746492) in the 3p21.31 genetic locus, which includes the CCL-2 receptor, *CCR2*, were associated with increased *CCR2* expression ($p = 8.2e\text{-}5$ and $5.2e\text{-}5$, respectively), decreased CCL-2 serum level ($p = 2.41e\text{-}9$ and $6.21e\text{-}9$, respectively), and increased risk of T1D ($p = 7.9e\text{-}5$ and $7.9e\text{-}5$, respectively).

The 3p21.31 genetic region is associated with developing T1D through regulatory control of the CCR2/CCL2 immune pathway.

1. Introduction

Type 1 diabetes (T1D) is an autoimmune disorder where some genetically predisposed individuals are exposed to an environmental trigger which leads to immune recognition and destruction of pancreatic islet cells [1]. T1D is associated with microvascular and macrovascular complications and a shortened lifespan [2,3]. A primary goal in T1D is primary prevention of the disease. Recent success in anti-CD3 inhibition delaying progression from islet autoantibody positivity to T1D has brought promise to immunomodulation for T1D prevention [4]. The use of genome wide association studies (GWAS) may help identify other immune targets [5,6].

GWAS have associated the 3p21.31 locus with T1D and pediatric autoimmune diseases (including T1D) [6,7]; however, the causal variants and mechanism of association have not been established for this locus. This region of the human genome is enriched for chemokine receptors that play an important role in autoimmunity [8], including the C–C motif chemokine receptors (*CCR1*, *CCR2*, *CCR3*, *CCR5*, *CCR9*, *CCRL2*, *CXCR6*) and X–C motif chemokine receptor 1 (*XCR1*). Associated pathways include chromatin remodeling genes (*SETD2*, *ELP6*, and

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SMARCC1), putative transcriptional regulators (*CCDC12*, *CCDC51*, *CCDC36*, and *ZNF589*), and cell replication associated genes (*KIF9* and *CDC25A*) [9], as well as the *CCRL2-LINC02009* [6] and *DAG1* [7] loci.

CCR2 is a pro-inflammatory chemokine receptor which, upon binding with its ligand CCL-2, promotes effector T-cell activation [10] and recruitment of M2 macrophages [11,12]. Non-obese diabetic (NOD) *CCR2−/−* mice develop T1D at a slower rate than NOD wild-type mice [13]. Serum CCL2 levels are different between T1D and controls in case-control studies; however, the direction of difference is contested [14–16]. Lower serum levels of CCL-2 have been reported in children with persistent islet autoantibodies compared to controls [17], suggesting a potential causative role of *CCR2* in T1D pathogenesis [18,19]. *CCR2* has also been implicated in other immune diseases, including rheumatoid arthritis (RA) [20–22] and multiple sclerosis (MS) [23–25]. However, clinical trials of CCR2 antagonists did not show reduce disease progression in either RA or MS patients [26,27].

CCR2 inhibition is associated with increased serum levels of its ligand, CCL2 or MCP-1, in both mouse and human studies [26,28,29]. CCR2 activation by CCL2 has been shown to cellular uptake and internalization of the CCR2/CCL2 complex [30]. A recent GWAS identified SNPs near *CCR2* in the 3p21.31 genetic locus associated with serum CCL2 levels. These studies suggest that *cis*-acting genetic variants which alter CCR2 expressivity could paradoxically change serum CCL2 levels [31].

In this report, the direction of the association between CCL2 level and T1D progression is determined in a longitudinal cohort (DAISY) and potential causal variants in the 3p21.31 locus were identified.

2. Methods

Human subjects: The prospective cohort was part of the DAISY study [32,33]. The DAISY study recruited newborns at high risk of developing T1D, defined as newborns with first degree relatives diagnosed with type 1 diabetes and newborns with high risk HLA genotypes for type 1 diabetes. The sex, age, family history of T1D, and HLA-DQB1 genotypes for all subjects were summarized in Table 1. Serum samples were collected in clot activator tubes and processed and stored within less than 1 h. Repeated freezer/thawing cycles were avoided for all samples. Islet autoantibodies to insulin, GAD, IA-2 and ZnT8 were measured by radio-binding (RBA) assay. Persistence was defined by presence of at least one of the autoantibodies on at least two consecutive visits 3–6 months apart.

Laboratory assays: Serum levels of CCL-2 were measured using multiplex fluorescent immunoassays (Millipore, Billerica MA) according to the manufacturer's recommendations. The coefficients of variation (CV, <10%) and the minimum detectable dose (MDD, CCL-2: 0.9) for the assays were provided by the manufacturer with the assay kits.

Serum samples (5x diluted) were incubated with capture antibodies immobilized on polystyrene beads for 1 h. The beads were then washed and further incubated with biotinylated detection antibody cocktail for 1 h. Beads were washed twice to remove unbound detection antibody and then incubated with phycoerythrin-labeled streptavidin for 30 min. Prior to reading, the beads were washed 2 times and suspended in 60 μl of wash buffer. The median fluorescence intensities (MFI) were measured on a FlexMAP 3D array reader (Millipore, Billerica, MA) using the following instrument settings: events/bead: 50, minimum events: 0, flow rate: 60 μl/min, Sample size: 50 μl and discriminator gate: 8000–13500. Before profiling, the serum dilutions were optimized by performing the assays at different serum dilutions to ensure that the majority of the data falls within the linear range of the standard curve.

Protein concentrations were estimated using a regression fit to the standard curve with known concentration included on each plate using a serial dilution series. The data were subjected to several quality control steps before further analysis [14].

Data acquisition: DAISY phenotype data was provided by MR. CCL-2 pQTL data were downloaded from Zenodo (https://zenodo.org/record/2615265#.YGs9GEhKjYk) on January 05, 2021. The data were generated by the Systematic and Combined AnaLysis of Olink Proteins (SCALLOP) consortium which measured 90 serum proteins associated with cardiovascular function in 30, 931 individuals. The individual sera were from 13 separate European cohorts, which each performed sample genotype using a SNP array and performed imputation to the 1000 Genomes Project phase 3 reference or later or to the Haplotype Reference Consortium reference. An average of 20.3 million SNPs were investigated for association with each of the 90 proteins. T1DGC summary statistics data were provided by SOG and SR [6]. The data were generated from T1DGC by typing 135,870 custom selected SNPs on 18,892 subjects using the Illumina Infinium high-density genotyping array, ImmunoChip (Illumina, Inc; CA). The data is a meta-analysis of a traditional case-control GWAS and a association study based on 2601 T1D affected sib pair (ASP) families from Asia-Pacific, Europe, North America, and the United Kingdom. CCR2 eQTL data were downloaded from GTEx portal (https://www.gtexportal.org/home/gene/CCR2) on January 05, 2021. The GTEx data were generated from ~1000 individuals with genotyping on the HumanOmni5M-Quad BeadChip and the Illumina Infinium HumanExome BeadChip from donor blood. RNA sequencing was performed with the Illumina Hiseq 200 using a 76-base, paired-end Illumina TruSeq RNA protocol.

Statistical analysis: Statistical analyses of serum CCL2 levels were performed using SAS software version 9.4. A p-value less than 0.05 was considered significant. Because CCL-2 values are not normally distributed, Box-Cox transformation was performed to normalize the data. The optimal power parameter (lamda) was obtained using PROC TRANSREG by using a maximum likelihood criterion [34]. The optimal power parameter (Lamda) was found to be 0.75 and tCCL-2=((CCL-2)**0.75–1)/0.75. Linear mixed models (PROC mixed) are used to compare the difference in Box-Cox transformed CCL-2 levels among the three study groups, adjusting for age, gender (female vs male) and HLA genotype ([DR3/4 or DR4/4 or DR4/X] vs [DR3/3 or DR3/X or DRX/X]) and FDR status (FDR vs GP) with random intercept in the model [35]. Fit statistics between different curves over age were compared for a lower AIC/BIC and the model with quadratic curve over age was chosen as the final model.

Additional statistical analyses were performed using the R language and environment for statistical computing (R version 4.0.5; R

Table 1
Characteristics of study participants by study group. Categorical variables were analyzed using Pearson $\chi 2$ tests. Continuous variables were tested using the *t*-test for differences in means or the Wilcoxon rank sum test for differences in medians.

		Controls N = 220	Non-progressors N = 42	Progressors N = 48	P value
Age of the first sample, median (IQR)		1.3 (0.8–2.4)	1.3 (0.8–3.1)	1.3 (0.8–2.6)	0.68
Number of serum samples per subject, median		7 (5–10)	11 (7–16)	14 (10–17)	<0.0001
HLA genotype, N (%)	DR3/3 or DR3/X or DRX/X	89 (40.0)	13 (31.0)	12 (25.0)	0.09
	DR3/4 or DR4/4 or DR4/X	131 (60.0)	29 (69.0)	36 (75.0)	
Fist-degree relative, N (%)	No	94 (42.7)	16 (38.1)	17 (35.4)	0.60
	Yes	126 (57.3)	26 (61.9)	31 (64.6)	
Sex, N (%)	Female	96 (43.6)	22 (52.4)	24 (50.0)	0.48
	Male	124 (56.4)	20 (47.6)	24 (50.0)	

Foundation for Statistical Computing; www.r-project.org). PLINK1.9 [36] was used for pre-processing the genotype data. LocusZoom [37] was used to generate both Manhattan and LocusZoom plots. All p-values were two–tailed and a $p < 0.05$ after adjusting for multiple testing was considered statistically significant.

Data and Resource Availability: The CCL-2 serum level data DAISY are available upon request to the authors. T1DGC summary statistics data were provided by SOG and SR [6] and are available upon request. CCL-2 pQTL data are available from Zenodo (https://zenodo.org/record/2615265#.YGs9GEhKjYk). CCR2 eQTL data are available from GTEx portal (https://www.gtexportal.org/home/gene/CCR2).

3. Results

3.1. Serum CCL-2 levels decrease over time in DAISY participants who develop T1D compared to controls

In this study, we measured CCL-2 levels in longitudinally collected serum samples from 310 children at high risk for T1D participating in the Diabetes Autoimmunity Study in the Young (DAISY) [32,33]. At the time of analysis, 48 DAISY participants developed persistent islet autoantibodies and progressed to T1D (progressors) and an additional 42 participants developed persistent islet autoantibodies but have not progressed to T1D (non-progressors). Their longitudinal CCL-2 levels were compared to those of 220 islet autoantibody negative children (controls) similar to the cases in terms of age, sex, family history of T1D and HLA genotypes (Table 1).

The subjects were followed for 16.80 years on average (range 1.98–31.00 years). On average, 9.34 (range 3–30) serial serum samples were collected from each subject. The mean age at the appearance of islet autoantibodies was 4.6 years in progressors and 7.5 years in non-progressors. The mean age of T1D diagnosis in progressors was 12.2 years.

CCL-2 was detectable in all samples from the 310 DAISY subjects. CCL-2 levels decreased with age in all groups. We next tested whether the average CCL-2 levels differed among the three study groups, using linear mixed models adjusting for age, sex, BMI [38], HLA genotype, and FDR status (Table 2). CCL-2 levels were significantly lower in both progressors ($p = 0.004$) and non-progressors ($p = 0.005$), compared to controls (Supplemental Figure 1). Subjects with high-risk HLA genotype (DR3/4 or DR4/4 or DR4/X) had lower MCP1 level compared to subjects with moderate-risk HLA genotype DR3/3 or DR3/X or DRX/X ($p = 0.009$). However, CCL-2 levels did not differ by sex or by the presence of a first-degree relative.

Table 2
Multivariate mixed models analysis of the CCL-2 levels.

Effect		Estimate	Standard Error	Pr > |t|
Intercept		87.44	2.42	<.0001
Age*Age		−0.01	0.01	0.17
Sex	Female vs Male	−0.79	1.94	0.68
HLA genotype	DR3/4 or DR4/4 or DR4/X vs DR3/3 or DR3/X or DRX/X	−5.82	2.11	0.006
First-degree relative with T1D	Yes vs No	1.48	2.06	0.47
BMI Z-score		0.24	0.82	0.77
Study Group	Non-progressors vs Controls	−7.43	2.73	0.007
	Progressors vs Controls	−6.33	2.57	0.014
	Progressor vs Non-progressors	1.10	3.33	0.74

3.2. The 3p21.31 genetic locus is associated with serum CCL-2 levels

Genetic variants associated with serum CCL-2 levels were determined by downloading summary statistics from a study estimating *cis*- and *trans*-protein quantitative trait loci (pQTLs) for 90 proteins using over 20,000 subjects [31]. One locus contributing to serum CCL-2 levels at 3p21.31 was identified (Fig. 1A), centered around the *CCR2* gene (Fig. 1B). The SNPs on chromosome 3 accounted for ~10% of the variation in CCL-2 serum level, which explains 70% of estimated heritability [31]. An additional study in the GWAS catalog [39] validated the association between the 3p21.31 locus and serum CCL-2 levels [40].

3.3. Potential causal variants for serum CCL-2 level regulation and T1D pathogenesis

Since the 3p21.31 locus is associated with both CCL-2 serum levels and T1D, and CCL-2 serum levels are associated with T1D, genetic variants associated with T1D through regulatory control of CCL-2 serum levels were targeted for evaluation. Multiple levels of data were integrated to identify a potential set of causal variants from the 3p21.31 locus. Summary statistics were obtained from the T1DGC ImmunoChip fine mapping data [6], which profiled 135,870 custom selected SNPs on 18,892 subjects. After merging the summary statistics from CCL-2 protein quantitative trait loci (pQTL) and T1DGC data, a total of 8804 SNPs on chromosome 3 overlapped both the CCL-2 and T1DGC studies, including 576 SNPs between position chr3:46,300,000–46,800,000 (Fig. 1C). Using a Bonferroni-adjusted p-value cut-off, 225 SNPs were associated ($p < 8.7 \times 10^{-5}$) with CCL-2 serum levels, with 63 SNPs associated with T1D. A total of 47 SNPs were associated with both T1D and CCL-2 serum levels. Expression QTLs (eQTLs) associated with *CCR2* were obtained from the Genotype-Tissue Expression (GTEx) portal for whole blood. Eighty-six SNPs overlapped the combined CCL-2 pQTL and T1DGC fine mapping data and the GTEx whole blood eQTL data at chr 3:46,300,000–46,800,000 (Fig. 1D).

Two SNPs, rs1799988 and rs746492, were significantly associated with T1D, CCL2 serum pQTL, and *CCR2* eQTLs (Fig. 2), suggesting that the two SNPs could play a regulatory role in CCR2/CCL2 pathway activation and progression to T1D. We therefore propose a mechanism linking the two potential causal variants to T1D pathogenesis. Individuals with these variants have associated increased immune cell expression of *CCR2* and the associated increase in CCR2/CCL2 signaling, which causes immune-mediated islet destruction and depletion of serum CCL-2 levels (Fig. 3).

4. Discussion

While some studies have shown that serum CCL-2 levels are increased in T1D subjects compared to normal controls, our previous work and current findings do not agree with this [14–16]. By analyzing serum CCL-2 levels in a longitudinal cohort, we were able to demonstrate the decrease in serum CCL-2 levels as subjects progressed from autoantibody positivity to T1D (Table 2). The decrease in serum CCL-2 with disease progression is in agreement with our proposed mechanism. *Cis*-acting genetic variants increase *CCR2* expression above the average population level which increases CCR2/CCL2 axis signaling and immune cell recruitment to the pancreatic islet. The increased signaling depletes the serum pool of CCL2 (Fig. 3). These findings contradict the simple logic that increased serum inflammatory markers indicate a pro-inflammatory state. A similar mechanism has been described where decreased IL-6 serum levels are associated with worse cardiovascular prognosis due to increased IL-6R/IL-6 signaling in the vascular wall [41,42]. Our findings provide an additional example of decreased serum levels of a pro-inflammatory molecule associated with an increased inflammatory state.

Interestingly, serum CCL2 levels were more likely to be elevated at 3–4 months in infants who will develop islet autoimmunity in The

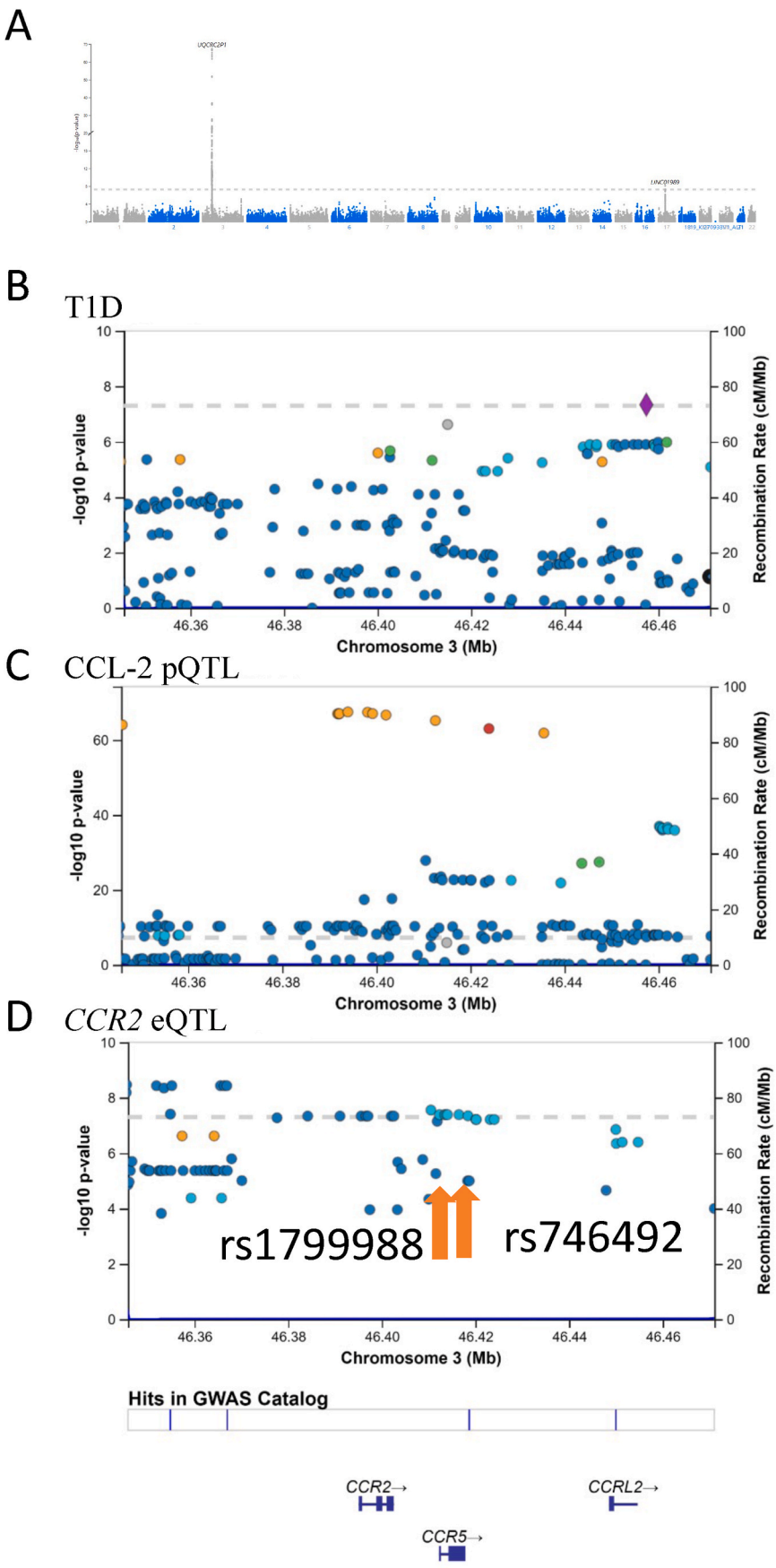

Fig. 1. GWAS for CCL-2 serum levels and fine mapping for type 1 diabetes. A) Manhattan plot of the serum CCL-2 protein quantitative trait loci (pQTL) from Folkersen et al. [31]. Data were downloaded from Xenodo and plotted using the LocusZoom plot package. The genome wide significant SNPs are from the 3p21.31 region. B) LocusZoom plot of SNPs significance on T1D association testing from T1DGC fine mapping study. C) LocusZoom plot of SNPs significance on CCL-2 pQTL from Fig. 1A. D) LocusZoom plot of SNPs significance on CCR2 eQTL from GTEx. The potential causal variants rs1799988 (chr3:46412259) and rs746492 (chr3:46417312) are indicated with arrows.

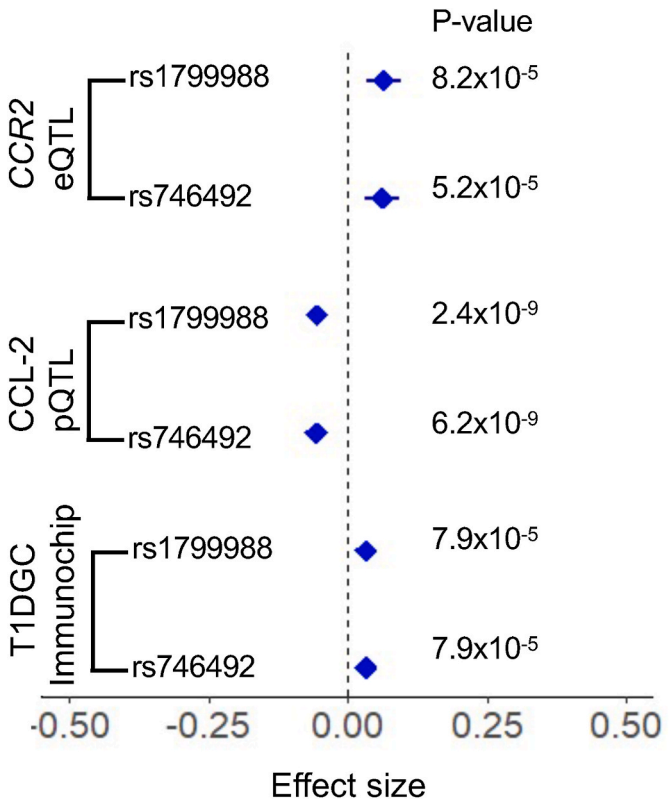

Fig. 2. Forest plot of potential causal SNPs (rs1799988 and rs746492) *CCR2* eQTL, CCL2 pQTL, and T1D Immunochip Association. The axis represents effect size with bars showing 95% confidence interval.

Environmental Determinants of Diabetes in the Young [43] (TEDDY) high risk genetic cohort (M. Rewers, personal communication). This may be due to early enterovirus infection, which has been shown to increase type 1 interferon and CCL2 expression [44]. A lack of statistical significance of decreased CCL2 levels in progression to islet autoimmunity and type 1 diabetes may be due to lack of statistical power in this cohort since 1) less samples were collected after seroconversion and 2) the cohort is a genetically pre-select group which may lack the traditional variation in CCL2 levels.

We were able to narrow the list of potential causal variants by integrating the CCL-2 pQTL and T1D Immunochip fine mapping. Although this integration provides high confidence for these SNPs, they will require functional validation. Additionally, while the T1D fine mapping study used a custom SNP array for autoimmune diseases [45], the CCL-2 GWAS and the GTeX eQTL study [46] used a broader SNP array. Thus, SNPs which were not assessed in all SNP arrays could not be fairly evaluated and may still regulate the CCR2/CCL2 axis.

Our findings support the potential of CCR2 inhibition in delaying the progression of islet autoantibody positive individuals to T1D. CCR2 inhibition could impair the recruitment of immune cells, including T cells and monocytes, to the pancreatic islet, and thus, delay the progression to islet autoimmunity. Several CCR2 inhibitors have been tested in clinical trials for cardiovascular disease, diabetic nephropathy, cancer, and autoimmunity [47–51]. Within autoimmunity, CCR2 inhibition did not change disease progression for subjects with rheumatoid arthritis or multiple sclerosis. One potential explanation is that CCR2 inhibition was targeted too late in the disease course and that earlier targeting based on serology and before clinical manifestations would modulate clinical progression [52]. Additionally, individuals with high CCL-2 serum levels, indicating a normal or low expression and activity of CCR2 would likely not benefit as much as individuals with high *CCR2* expression and activity with lower CCL-2 serum levels. Thus, potential future trials for T1D prevention should target autoantibody positive individuals with lower serum levels of CCL-2.

In conclusion, we mapped the T1D association of the 3p21.31 locus to the *CCR2* gene. Increased *CCR2* gene expression decreases serum levels of CCL-2 and is associated with increased immune destruction of pancreatic islets and quicker progression to T1D.

Credit author statement

Paul MH Tran: Conceptualization, Formal analysis, Data curation, Writing – original draft, Visualization **Sharad Purohit:**

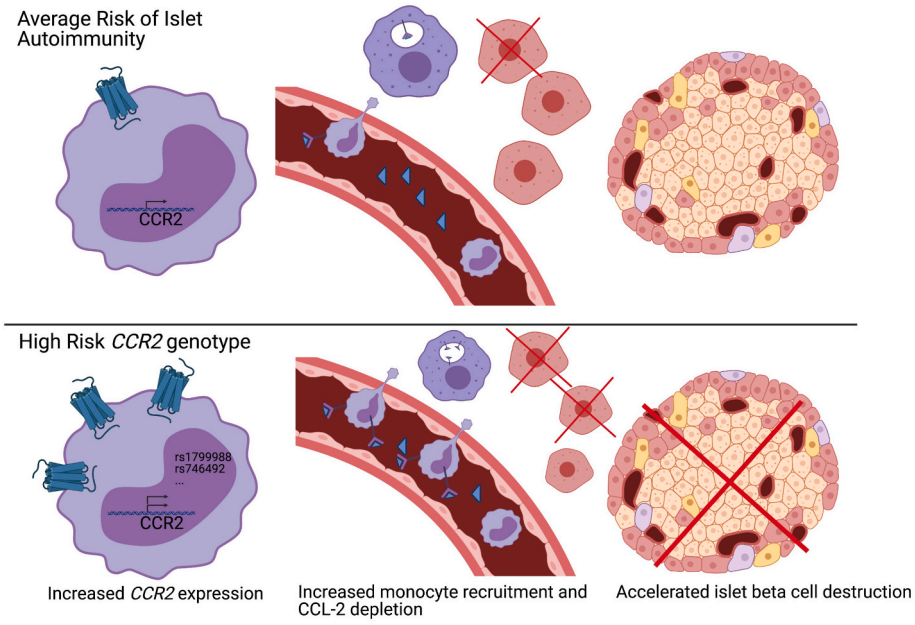

Fig. 3. Proposed model of 3p21.31 locus influence on progression to type 1 diabetes. Individuals with high risk *CCR2* genotypes are more prone to increased *CCR2* expression, increased CCR2/CCL2 axis signaling with subsequent depletion of serum CCL2, increased monocyte recruitment, and increased rate of pancreatic islet destruction, resulting in an increased rate of progression to type 1 diabetes. Created with BioRender.com.

Conceptualization, Methodology, Resources, Data curation, Visualization, Funding acquisition Khaled bin Satter Data Curation, Visualization **Eileen Kim:** Data Curation, Writing- Review & Editing **Fran Dong:** Formal analysis, Writing – review & editing **Kathleen Waugh:** Data Curation, Writing- Review & Editing **Diane Hopkins:** Data Curation **Marian Rewers:** Resources, Data curation, Supervision, Writing- Review & Editing **Suna Onengut-Gumuscu:** Resources, Data curation, Writing – review & editing **Stephen S Rich:** Data Curation, Resources, Funding acquisition **Jin-Xiong She:** Supervision, Resources, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Dr. Jin-Xiong She is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtauto.2021.100127.

Author contributions

PMHT wrote the manuscript and researched data. SP acquired the Luminex data; SP, KBS, EK, PMHT, FD, KW and MR were responsible for data analysis. DH, KW, MR and JXS contributed to clinical samples. S O-G and SSR generated fine mapping data. All authors contributed to writing and editing of the manuscript.

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