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Iddm30 Controls Pancreatic Expression of Ccl11 (Eotaxin) and the Th1/Th2 Balance within the Insulitic Lesions

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The autoimmune diabetic syndrome of the BioBreeding diabetes–prone (BBDP) rat is a polygenic disease that resembles in many aspects human type 1 diabetes (T1D). A successful approach to gain insight into the mechanisms underlying genetic associations in autoimmune diseases has been to identify and map disease-related subphenotypes that are under simpler genetic control than the full-blown disease. In this study, we focused on the β cell overexpression of diabetogenic in BBDR rats, a BBDP-related strain. We tested the hypothesis that this trait is genetically determined and contributes to the regulation of diabetes in BBDR rats. Similar to the BBDR strain, we observed a time-dependent, insulin-dependent pancreatic upregulation of Ccl11 in BBDR rats when compared with T1D-resistant ACI.Iu.Iyp animals. Through linkage analysis of a cross-intercross of these two parental strains, this trait was mapped to a region on chromosome 12 that overlaps Iddm30. Linkage results were confirmed by phenotypic assessment of a novel inbred BBDP.ACI-Iddm30 congenic line. As expected, the Iddm30 BBDP allele is associated with a significantly higher pancreatic expression of Ccl11; however, the same allele confers resistance to T1D. Analysis of islet-infiltrating T cells in Iddm30 congenic BBDP animals revealed that overexpression of pancreatic Ccl11, a prototypical Th2 chemokine, is associated with an enrichment in Th2 CD4+ T cells within the insulin lesions. These results indicate that, in the BBDP rat, Iddm30 controls T1D susceptibility through both the regulation of Ccl11 expression in β cells and the subsequent Th1/Th2 balance within islet-infiltrating T lymphocytes. The Journal of Immunology, 2014, 192: 3645–3653.

The BioBreeding diabetes–prone (BBDP) rat is a model for human type 1 diabetes (T1D) (1). The diabetic syndrome of the BBDP rat is a polygenic disease characterized by an early infiltration of the pancreas also known as insulitis that precedes the T cell–mediated autoimmune destruction of insulin-secreting pancreatic β cells (2–7). In our rat colony, 80–90% of BBDR rats spontaneously develop T1D by the age of 120 d, without sex bias. Two T1D susceptibility genes have been previously identified in this animal model, as follows: the MHC (RT1) class II h haplotype (Iddm1) on chromosome 20 (3, 8, 9) and a recessive null mutation of GPIase, IMAP family member 5 (Gimap5; Iddm2; Lyp) on chromosome 4 (3, 10, 11). This Gimap5 mutation is responsible for the premature death of recent thymic emigrants, resulting in severe peripheral lymphopenia that affects all T cell subsets, including regulatory T cells (12, 13). This impaired development of regulatory T cells appears to be the main diabetogenic mechanism contributed by this mutation (12, 14, 15). Although multiple additional Iddm loci linked to either T1D, severity of insulitis, or age of disease onset were subsequently mapped in the BBDR rat, the chromosomal intervals of these loci were large, thus making the search for the causal genes within and the characterization of the underlying pathogenic mechanisms controlled by these genes difficult (2, 16, 17). A successful approach to obtain insight into the genetic basis of autoimmunity has been to identify and map disease-related subphenotypes that are highly penetrant, quantifiable, and under simpler genetic control than the multistep, full-blown autoimmune disease (3, 14, 18–21). The cloning of the lymphopenia-causing gene in the BBDP model is an example of this approach (22, 23). We focused in this study on a subphenotype that was recently observed at the level of the target tissue of the diabetogenic process, namely the upregulation of the chemokine Eotaxin (Ccl11) in the pancreatic β cells of T1D-prone rats (24).

The pancreatic β cell–specific upregulation of Ccl11 was first reported in DR rats, an inbred strain that was derived from the original BBDP/Worcester forebears (24). By using immunohistochemistry on pancreatic sections, Ccl11 was detected in pancreatic β cells as early as 40 d of age, hence prior to any detectable signs of insulitis in lymphopenic and T1D-susceptible DR/Iyp rats, and in age-matched, T1D-resistant, and insulin-free DR+/− rats that are congenic for wild-type Gimap5 (24). This β cell–specific expression of Ccl11 was, however, not detectable in T1D-resistant Wistar–Furth (WF) rats that share the same MHC RT1u haplotype (24). Together, these observations suggested that β cell overexpression of Ccl11 is an early preinsulitic phenotype that is independent of Iddm2 and possibly under control of the DR rat genetic background.

The observation of chemokine expression by β cells is not new, and, in fact, multiple chemokines are readily detectable in normal
rodent and human pancreatic islets (25, 26). The physiological significance of this expression remains elusive, although it has been shown that the interaction of extracellular matrix proteins with β cells results in NF-κB activation and the subsequent synthesis of cytokines by β cells, in vitro (27). Ccell is one of the three members of the eotaxin family of C-C chemokines that are potent chemoattractants for Ccrr3-expressing cells, which include eosinophils, mast cells, basophils, neutrophils, and Th2 lymphocytes (28–34). Although the presence of eosinophils and mast cells within the insulitic lesions of BBBD rats has been previously reported (35–37), the observation that Ccell is upregulated in the target organ of this diabetogenic process remains puzzling (24, 37), particularly because diabetes in the BBBD rat is a Th1-mediated autoimmune disease (38–40). Notwithstanding, Hessner’s group (37) examined the role of pancreatic Ccell expression in T1D pathogenesis in Dkhyp hypy rats. Their observation that therapeutic inhibition of mast cell degranulation delayed the onset of disease in these animals provided circumstantial evidence that elevated expression of pancreatic Ccell may be pathogenic (37).

In this study, we have tested the hypothesis that pancreatic overexpression of Ccell is genetically determined and contributes to the regulation of the diabetogenic process in the BBBD rat. We observed a differential pancreatic expression of Ccell between T1D-susceptible BBBD and T1D-resistant ACI.1u.lyp rat strains. Specifically, Ccell transcript levels were upregulated in a time-dependent fashion in the BBBD rat pancreas, and this upregulation was confirmed to be Iddm30 independent. Through linkage analysis of a cross-intercross of BBBD and ACI.1u.lyp strains, we mapped this quantitative trait to a region of chromosome 12 that overlaps with a previously identified locus, Iddm30 (2). These linkage results were confirmed through the development and subsequent phenotypic analysis of a BBBD strain congenic for the chromosome 12 interval that controls T1D, insulitis, and Ccell expression in pancreatic islets. Importantly, our data show that whereas the BBBD allele at Iddm30 is associated with the expected higher levels of pancreatic Ccell, this allele confers resistance to T1D. Further analysis of islet-infiltrating T cells in Iddm30 congenic BBBD animals revealed an increased proportion of IL-4 synthe-
sizing CD4⁺ T cells in rats homozygous for the BBBD allele. Taken together, our results strongly suggest that the elevated recruitment of these Th2 cells was driven by the pancreatic overexpression of Ccell and resulted in a shift of the intraislet Th1/Th2 balance toward Th2 that may have contributed to diabetes resistance.

Materials and Methods

Animals

The parental inbred BBBD and ACI.1u.lyp rat strains and the congenic lines derived from them have been maintained at Sunnybrook Research Institute under specific pathogen-free conditions. Inbred BBBD rats originally purchased from Biomedical Research Models (Worcester, MA) have been maintained for >30 generations of brother × sister breeding and are homozygous at every genetic marker tested across the whole genome. Nonlymphopenic, diabetes-resistant BBACI-Iddm2 rats (BB NON LYP) congenic for wild-type Gimap5 have been previously described (15). The inbred ACI.1u and ACI.1u.lyp strains were derived from the Augustin Copenhagen Irish (ACI)/Hsd rats (Harlan Sprague Dawley, Indianapolis, IN) through introgression of the BBBD RTu (Dknd1) locus alone or in combination with the BBBD Gimap5 (Lyp) locus (Iddm30) (2). To generate the (BBBD × ACI.1u)F1.lypF1 cohort, BBBD males were first crossed with ACI.1u.lyp females to generate F1 progenies that were then intercrossed for linkage analysis. The results of this linkage analysis for type 1 diabetes, severity of insulitis, and age of disease onset have been reported previously (2).

Development of congenic inbred lines of BBBD rats

For generation of the congenic inbred BBBD.ACIC-Iddm30 (BB.BC-Iddm30) line, a ∼36-Mb interval of ACI.1u.lyp chromosome 12 that encompasses the 1 log of odds (LOD) interval of Iddm30 as defined by F2 linkage analysis (2) was introgressed into the BBBD background. Specifically, BBBD and ACI.1u.lyp rats were first intercrossed, and this was followed by 10 successive, marker-assisted backcrosses to the BBBD strain. Progenies of the final backcross, which were heterozygous at Iddm30, were then intercrossed to fix the region as ACI homozygous. Diabetes-prone animals were tested for glycosuria and ketonuria three times per week after 60 d of age. Animals found positive were then tested for hyperglycemia and considered as diabetic if blood glucose was >16.6 mM. All animal protocols were approved by the Sunnybrook Animal Care Committee.

Genotyping and linkage analysis

Genotyping and linkage analysis were performed, as previously described (2). Briefly, diabetic F2 animals were genotyped across the genome using 229 microsatellite markers at an average distance of 12.5 Mb apart. Microsatellite genotyping was done using PCR amplification, and PCR products were separated and analyzed on 3% agarose gel for allelic determination. Physical locations and primer information of microsatellite markers were obtained from the Rat Genome Database (http://rgd.mcw.edu). Segregation analysis of T1D based on comparison of the observed to expected Mendelian inheritance genotype distribution at each marker was then performed, and markers linked to diabetes with a LOD score >1 were considered as diabetes associated. Further refinement of linkage was obtained through genotyping of additional markers flanking these peaks of linkage, followed by interval mapping analysis.

RNA extraction from pancreatic tissue and quantitative RT-PCR

Total RNA was isolated from carefully dissected and immediately homogenized rat pancreas using GenElute mammalian total RNA miniprep kit (Sigma-Aldrich, St. Louis, MO). RNA samples were treated with DNase I (Life Technologies, Green Island, NY) to eliminate genomic DNA contamination prior to first-strand cDNA synthesis using oligo-dT primer (Fisher Scientific, Pittsburgh, PA) and Superscript III (Life Technologies) according to manufacturer’s instructions. Quantitative real-time PCR (qRT-PCR) was performed using an ABI Prism 7000 sequence detection system (Life Technologies). PCR consisted of iTaq SYBR Green supermix with ROX (Bio-Rad, Hercules, CA), β-actin primers (forward, 5'-CCC TAA GGC CAA CCG TGA A-3'; reverse, 5'-ACC AGA GGC ATA CAG GGA CAA-3'; Sigma Genosys) as quantification standard, and Ccell primers (forward, 5’-CAG TTC TCC ACA GCA CCT CT-3’; reverse, 5’-CTG GTC ATG GTA AAC CGG CA-3’; Sigma Genosys). Triplicate reactions were done for each sample in 20 μL reactions. Specificity for qRT-PCR amplification was verified by melting-curve analysis and through detection of a single PCR product on 3% agarose gel. Data were analyzed on ABI Prism 7000 software using the ΔCt cycle threshold (ΔCt) method to calculate the relative transcript copy number of Ccell to β-actin. Unless otherwise stated, t test was used for all statistical analyses and a p value <0.05 was considered as significant.

Immunofluorescent staining of pancreatic sections

Two-color immunofluorescent staining of pancreatic sections for rat Ccell and pancreatic hormones used goat polyclonal anti-Ccell (R&D Systems, Minneapolis, MN) and mouse monoclonal anti-insulin or anti-glucagon Abs (Sigma-Aldrich), and was performed as described previously (37). To briefly, freshly excised pancreas were fixed in 10% phosphate-buffered formalin (Cedarlane Laboratories, Burlington, ON, Canada) at 4°C overnight. The following day, tissues were dehydrated by serial incubations in 10, 20, and 30% sucrose solutions. Samples were then embedded in optimal cutting temperature medium (Electron Microscopy Sciences, Hatfield, PA) and snap frozen. Two 4-μm sections cut 250 μm apart were processed from each pancreas and mounted onto t-lysine-coated and mounted onto t-lysine-coated microscope slides. Pancreatic sections were first subjected to Ag retrieval using citrate-based Ag retrieval solution (Dako, Carpinteria, CA) at 95°C for 10 min, then rinsed in Tris buffer (pH 7.6), followed by blocking with 5% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA). Sections were stained overnight in a humidity chamber with appropriate dilutions of primary Ab combinations. Sections were then rinsed and stained with the appropriate secondary Abs, Texas Red–conjugated donkey anti-goat IgG and FITC-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories), for 1 h at room tempera-
ture. Slides were washed, dried, and mounted using fluorescence mounting medium (Dako). Slide analysis was done on a Zeiss Axiovert inverted fluorescent microscope using Apotome optical sectioning imaging system (Zeiss, Thornwood, NY).
Assessment of Ccl11 protein levels in isolated pancreatic islets by immunoprecipitation and Western blot

Pancreatic islets were isolated from age-matched, nonlymphopenic, and T1D-resistant BB.NonLyp and ACI.1u, as previously described (41). In brief, whole pancreas was distended by collagenase D (Sigma-Aldrich) and digested to homogeneity at 37°C. Digestion was stopped by adding ice-cold RPMI 1640 medium containing 10% FBS (Sigma-Aldrich). Total tissue suspension was then filtered through a 400-μm nylon filter and resuspended in ice-cold fresh RPMI 1640. The dissociated islets were handpicked under a dissection microscope. To maximize the purity of the islet preparation, the handpicking was repeated two more times and the final purity was verified by staining a portion of the isolated islets with 0.01% w/v diethiane (Sigma-Aldrich) in 10 mM HEPES-buffered PBS solution (pH 7.2). Islets were then lysed using Nonidet P-40 solution (Nonidet P-40, Sigma-Aldrich, Indianapolis, IN) lysis buffer with Complete Protease Inhibitor (Roche), and the total protein concentration was determined by bicinchoninic acid assay (Life Technologies). Equal amounts of islet-derived protein extract were incubated with protein A–Sepharose beads (Sigma-Aldrich) pre-coated with rabbit polyclonal anti-rat Ccl11 Ab (Peprotech, Rocky Hill, NJ) for immunoprecipitation (IP) at 4°C overnight. To establish the semiquantitative nature of the IP-Western blot assay for Ccl11 in ex vivo islets, we performed the same assay on positive controls.

Pancreatic transcript levels of Ccl11 are linked to Iddm30

Pancreatic islets were isolated from nondiabetic, age-matched, congenic inbred BB.DDR and BB.ACI-Iddm30 animals, as described above. The isolated islets were washed once in PBS with 1 mM EDTA and then digested in 0.25% Trypsin-EDTA (Life Technologies) at 37°C for 15 min. Digestion was stopped by adding ice-cold IMDM medium containing 10% FBS, and mononuclear cells were then isolated using a discontinuous Percoll gradient (GE Life Sciences, Pittsburgh, PA). Mononuclear cells were cultured in IMDM containing 10% FBS in the presence of 25 ng/ml PMA, 250 ng/ml ionomycin (Sigma-Aldrich), and brefeldin A (eBioscience, San Diego, CA) for 3 h. Activated mononuclear cells were then stained with CD4-specific, allophycocyanin-conjugated, W3/25 mAb (Sunnybrook mAb core facility, Toronto, ON, Canada), permeabilized using BD Cytofix/Perm kit, and stained for intracellular cytokines using IFN-γ-specific, FITC-conjugated, DB-1 mAb (BioLegend, San Diego, CA) and IL-4-specific, PE-conjugated, OX81 mAb (BioLegend). Stained cells were analyzed by flow cytometry using a FACSCalibur cell analyzer (BD Biosciences), and the proportions of IFN-γ and IL-4–specific CD4+ T cells were determined using FlowJo software.

Results

We sought to determine whether, similar to the observation made between diabetes-prone DR and diabetes-resistant WF strains, Ccl11 was differentially expressed in the pancreatic islets of diabetes-prone BB.DDR and diabetes-resistant ACI.1u.lyp animals. If this were the case, we reasoned that we would be able to take advantage of an ongoing cross-intercross between these two parental strains for concurrent linkage analysis of this trait and T1D, whereby allowing us to determine whether pancreatic Ccl11 expression is under genetic control and linked to diabetes.

Overexpression of Ccl11 in the pancreatic β cells of BB.DDR rats

Using two-color immunofluorescence for Ccl11 and pancreatic hormones, we observed that Ccl11 was detectable in the β cells of virtually all islets from 90-d-old BB.DDR rats (n = 10). A representative pattern of immunofluorescence is illustrated in Fig. 1A and 1B. However, a similar pattern of expression was observed in age-matched ACI.1u.lyp animals (n = 10) as well as in the non-lymphopenic, Gimap5 (Iddm2) congenic ACI.1u and BB.NonLyp strains (n = 8–10; data not shown). These results suggested that this trait was shared among the BBDR, BB.DDR, and ACI strains in an Iddm2-independent manner. To confirm this apparent phenotypic similarity in a more quantitative fashion, we assessed the levels of Ccl11 in lysates of adult pancreatic islets. To avoid any potential influence of ongoing islet inflammation in T1D-prone BB.DDR rats, this evaluation was performed in islets from non-lymphopenic, age-matched, adult BB.NonLyp and ACI.1u donors that are both T1D and insulin resistant. The low levels of Ccl11 protein expression required enrichment of the islet extracts by specific IP prior to quantification by Western blot. Validation of this quantitative evaluation was obtained by submitting serial dilutions of lysates from 293T cells transfected with rat Ccl11 to the same assay, as illustrated in Fig. 1C. Furthermore, incomplete depletion of Ccl11 by IP of the original lysates was ruled out by subjecting the supernatants from the first round of IP to an additional round of IP and Western blot for Ccl11 (Fig. 1D). Densitometry consistently showed a ~2-fold increase in Ccl11 content of pancreatic islets from BB.NonLyp rats compared with their ACI.1u counterparts (Fig. 1D). This differential expression of Ccl11 in the β cells of strains other than DR and WF further supports the notion that this trait is genetically determined. Furthermore, its overexpression in diabetes-prone DR and BB.DDR strains compared with diabetes-resistant WF and ACI animals, respectively, raises the possibility that its regulation plays a role in disease pathogenesis.

Quantitative assessment of islet Ccl11 expression by IP and Western blot is not applicable to a large cohort of animals required for genetic analysis. To circumvent this limitation, we sought to determine whether Ccl11 protein levels in pancreatic islets correlated with those of gene transcription from the whole pancreas using qRT-PCR analysis, a technique that would be amenable to higher throughput for linkage analysis. As illustrated in Fig. 2A, there was a ~6-fold increase in pancreatic transcript levels of Ccl11 in adult BB.DDR rats when compared with age-matched ACI.1u.lyp animals (p = 0.0002). This difference was similar to that observed between the pancreata of adult BB.NonLyp and ACI.1u donors (p = 0.0004). Meanwhile, no difference was found between Iddm2 (Gimap5) congenic BB.DDR and BB.NonLyp or ACI.1u.lyp and ACI.1u animals, confirming that this quantitative trait is not under the control of the Iddm2 locus. These differential transcript levels of Ccl11 also appear to be tissue specific, as Ccl11 transcripts were below the qRT-PCR detection threshold in liver and gut extracts collected from either strains. Next, we sought to determine whether this differential regulation of Ccl11 transcription between BB.DDR and ACI.1u.lyp rats was age dependent. As shown in Fig. 2B, significantly higher transcript levels of Ccl11 were found in the pancreas of BB.DDR donors as early as 30 d of age (p = 0.011), hence long before the onset of islet inflammation in these animals, and this difference between the two strains grew progressively larger over time. Having demonstrated that pancreatic expression of Ccl11 is differentially regulated between T1D-prone BB.DDR and T1D-resistant ACI.1u.lyp animals in a time-dependent fashion, we then sought to determine whether this quantitative trait is genetically determined through linkage analysis of a previously described F2 cohort that was derived from these two parental strains to map novel Iddm loci (2).

Pancreatic transcript levels of Ccl11 are linked to Iddm30

The assessment of Ccl11 transcript levels in the pancreas of BB(DDR × ACI.1u.lyp)F2 animals was initiated when the development of this cohort was well underway. F2 animals (n = 574) were followed prospectively until the onset of T1D (n = 127) or up to 165 d for nondiabetic animals. Of the 447 animals that remained
To control the severity of insulitis and T1D susceptibility in the whole heterozygotes (p significantly higher levels than ACI homozygotes (p = 0.0007) and heterozygotes (p = 0.0007). In contrast, there was no significant difference in Ccl11 transcription levels between animals that were ACI homozygous and heterozygotes (Fig. 3B). Indicating a recessive mode of inheritance for this trait.

Strikingly, the chromosome 12 locus influencing pancreatic transcript levels of Ccl11 overlaps with the Iddm30 locus that was found to control the severity of insulitis and T1D susceptibility in the whole cohort of 574 (BBDP × ACI.1u.lyp)F2 animals (Fig. 3A) (2). The 117 nondiabetic animals assessed for transcript levels of Ccl11 in the pancreas were derived from the last 167 members of the whole (BBDP × ACI.1u.lyp)F2 cohort, of which 45 (26.9%) became diabetic. The original report that Ccl11, a chemotactic factor for mast cells, was overexpressed in the pancreatic islets of T1D-prone DR rats compared with WF animals led to the hypothesis that this trait was diabetogenic (24), and this hypothesis was further supported by the observation by the same group that inhibition of mast cell degranulation slowed down the diabetogenic process (37).

In this context, it was important to determine whether at D12Rat51, where the BBDP genotype is associated with high levels of Ccl11 pancreatic transcripts in the (BBDP × ACI.1u.lyp)F2 cohort, the same genotype would also confer a high risk for T1D. As shown in Fig. 3C, this was not the case. Specifically, F2 animals that were BBDP homozygous at D12Rat51 exhibited a profound resistance to T1D with a cumulative incidence of only 8.5% by 165 d compared with 30.3 and 40% observed in heterozygotes and ACI homozygotes, respectively (p = 0.006 and p = 0.0004, respectively, by Kaplan–Meier survival analysis; Fig. 3C). Of note, similar to the levels of Ccl11 expression, the susceptibility to T1D controlled by the chromosome 12 locus appears to be inherited as a recessive trait. Taken together, these results indicate that the regulation of Ccl11 expression in the pancreas is under the control of a chromosome 12 locus that overlaps to a large extent with Iddm30, and the high levels of pancreatic Ccl11 expression are associated with protection from T1D.

The phenotypic analysis of BBDP rats congenic for Iddm30 confirms linkage of T1D and pancreatic Ccl11 expression to this locus

To confirm the results of linkage analyses, an inbred BBDP Iddm30 congenic line named BB.ACI-Iddm30 was developed.
transcripts in their pancreas. This parameter was assessed in Iddm30 resistance to T1D. homozygosity for the BBDP allele at Iddm30

Increased levels of Ccl11 expression in the pancreas are associated with a Th2 bias among CD4+ T cells present in insulitic lesions

T1D is considered as a prototypical Th1-mediated disease (38–40), and it has been demonstrated that Th2 skewing of the anti-pancreatic autoimmune response in diabetes-prone animals can result in disease protection (19, 43, 44). Increased expression of Ccl11 typically contributes to the recruitment of Th2 cells that express Ccr3, the chemokine receptor for Ccl11, to the site of inflammation (28, 32, 34, 45). The observation that high levels of pancreatic Ccl11 transcripts are associated with resistance to T1D in BBDB rats led us to determine whether these high levels are also correlated with a Th2 bias in the insulitic lesion of these animals. As illustrated in Fig. 5A, the transcript levels of the Th2 transcription factor Gata3 were significantly higher in the pancreas of 90-d-old BBDB rats (15.1 ± 3.6/10^3 β-actin copies, mean ± 1 SD) when compared with age-matched BB.ACI-Iddm30 animals (8.5 ± 2.9/10^3 β-actin copies, mean ± 1 SD; p = 0.0087). Next, we isolated the lymphocytes infiltrating the pancreatic islets of these animals to directly assess the proportion of CD4+ T cells synthesizing IFN-γ and IL-4 by intracytoplasmic staining and flow cytometry. The proportions of CD4+ T cells among the mononuclear cells isolated from the islets of nondiabetic, 90-d-old BBDB (n = 6) and BB.ACI-Iddm30 (n = 6) animals were not significantly different (26.8 ± 17.4% and 18.0 ± 10.8%, mean ± 1 SD, respectively; p = 0.3). Among these cells, the proportion of those synthesizing IFN-γ was also similar (26.2 ± 3.9%, mean ± 1 SD in BBBD versus 29.7 ± 6.5% in BB.ACI-Iddm30 rats; p = 0.5). In contrast, the proportion of CD4+ T cells synthesizing IL-4 among insulinic cells (Fig. 5B) recovered from BBBD animals (30.5 ± 8.4%, mean ± 1 SD) was significantly increased when compared with that from BB.ACI-Iddm30 donors (19.6 ± 5.5%, mean ± 1 SD; p = 0.02). Of note, histological analysis of pancreatic sections from diabetic and nondiabetic Iddm30 congenic BBDB animals revealed very low to undetectable numbers of mast cells within the pancreatic islets of both strains (data not shown). Taken together, our results demonstrate that homozgyosity for the BBBD allele of Iddm30 confers resistance to T1D and is associated with an increase in both pancreatic expression of Ccl11 and differentiation of insulitic CD4+ T cells toward the Th2 lineage.

Specifically, a <36-Mb interval of chromosome 12 of ACI.1u.lyp origin that encompasses the Iddm30 locus was introgressed into the BBDB genetic background (Supplemental Fig. 1). This congenic line was then followed prospectively for T1D and phenotypically assessed for transcript levels of Ccl11 in the pancreas.

When litters of BBDB and BB.ACI-Iddm30 animals were concomitantly followed for T1D development (Fig. 4A), the cumulative incidence of disease by 140 d was significantly higher in BB.ACI-Iddm30 rats (92%, n = 25) than in BBDB animals (75%, n = 36; p = 0.045 by one-tailed χ^2 test). Of note, the age of disease onset was not significantly different between the two Iddm30 congenic strains (78 ± 9.9 d in BBDB versus 82 ± 8.9 d in BB.ACI-Iddm30, mean ± 1 SD; p = 0.4 by Kaplan–Meier survival analysis). These results confirm that homozgyosity for the BBBD allele at Iddm30 confers significant resistance to T1D.

We then asked whether the increased susceptibility of BB.ACI-Iddm30 animals to T1D was associated with low levels of Ccl11 transcripts in their pancreas. This parameter was assessed in nondiabetic, age-matched ACI.1u.lyp and BBDB Iddm30 congenic animals. As illustrated in Fig. 4B, the levels of Ccl11 transcription were significantly higher in the pancreas of BBBD rats (456.5 ± 356.7/10^3 β-actin copies, mean ± 1 SD, n = 19) than in their Iddm30 congenic counterparts (116.3 ± 136.3/10^3 β-actin copies, mean ± 1 SD, n = 5; p = 0.011) at 90 d of age, whereas expression levels were similar between age-matched ACI.1u.lyp and BB.ACI-Iddm30 animals. Similar to what was observed in the parental strains, the pancreatic levels of Ccl11 expression in BB. ACI-Iddm30 animals increased with age (Fig. 4C). However, although these levels increased by almost an order of magnitude in BBBD rats between the ages of 90 and 150 d (456.5 ± 356.7/10^3 β-actin copies and 4286 ± 2633/10^3 β-actin copies, mean ± 1 SD, respectively), the corresponding elevation in ACI.1u.lyp and BB. ACI-Iddm30 animals was more modest, which explains the significant differences observed between BBBD rats and the two other strains at 150 d (353.7 ± 628/10^3 β-actin copies, mean ± 1 SD, n = 5 in ACI.1u.lyp, p = 0.0087 versus BBBD; 564.5 ± 513.9/10^3 β-actin copies, n = 9 in BB.ACI-Iddm30 rats, p = 0.012 versus BBBD). Thus, the analysis of Iddm30 congenic inbred animals confirms the linkage results whereby homozgyosity for the BBBD allele at Iddm30 is associated with both resistance to T1D and elevated levels of Ccl11 expression in the pancreas.

FIGURE 2. Increased Ccl11 mRNA levels in the pancreas of BBDB rats compared with ACI.1u.lyp animals. Ccl11 mRNA levels were assessed by qRT-PCR. The relative copy number of Ccl11 mRNA in each sample was normalized to the copy number of β-actin transcripts × 10^3 as determined by the dCT method. (A) Comparison of pancreatic Ccl11 transcript levels in 90-d-old BBDB, ACI.1u.lyp, and their respective Iddm2 (typ) congenic counterparts, BB.NonLyp and ACI.1u.lyp animals (individual values of each animal are shown; bars represent mean ± SEM; statistical significance was determined by t test, and significant p values (p < 0.05) are indicated unless no significance [n.s.] in difference was found). (B) Time-dependent upregulation of Ccl11 transcript levels in the pancreas of age-matched BBDB and ACI.1u.lyp rats. Four to twenty-three animals of each strain were analyzed at each time point (statistical significance was determined by t test; **p ≤ 0.01, ***p ≤ 0.001).
Discussion

Although there has been a plethora of studies characterizing alterations of the immune system that contribute to the loss of tolerance to pancreatic β cells in diabetes-prone individuals, very few have described intrinsic abnormalities of these endocrine cells that may play a role in the pathogenesis of the diabetogenic process. These include the upregulation of type I IFN and MHC class I expression in the β cells of diabetes-prone rodents prior to the detection of islet infiltration by myeloid and lymphoid cells (46, 47), and the evidence that the intense remodeling of pancreatic islets taking place around the time of weaning is dysreg-
mosome 12 that overlaps with one of the previously mapped T1D loci in the BBBDP rat, Iddm30 (2). However, we also provide evidence that this trait is associated not with increased susceptibility but rather with resistance to T1D and an increase in Th2 differentiation among the islet-infiltrating T lymphocytes.

The DR<sup>bhp</sup>bhp strain is genetically related to, yet exhibits genetic polymorphism with the inbred BBBDP strain (17). Despite this polymorphism, it has been demonstrated that these two strains share the same known susceptibility loci for spontaneous T1D (3, 14). Assuming that pancreatic overexpression of Ccl11 is genetically determined, the question followed as to whether this trait was controlled by one or more of the Iddm loci. If it were, the expectation was that we should be able to observe it in BBBDP animals. It was, however, unclear whether pancreatic expression of Ccl11 would differ between BBBDP and the T1D-resistant strain, ACI.1u.lyp, which we have previously used in genetic analyses.

Immunofluorescence analysis detected a similar expression of Ccl11 in the β cells of BBBDP and age-matched ACI.1u.lyp animals. However, the use of qRT-PCR and IP followed by Western blot both showed that this expression was significantly higher in the BBBDP strain at all time points analyzed, thus further supporting the notion that it is genetically determined. This differential expression between the BBBDP and ACI.1u.lyp strains was also observed in their Gimap5 (Iddm2) congenic counterparts, thereby confirming that pancreatic expression of Ccl11 is neither controlled by this locus nor secondary to islet inflammation. Analysis of a cohort of age-matched, non-diabetic (BBBDP × ACI.1u.lyp)F<sub>2</sub> animals showed significant linkage of pancreatic expression of Ccl11 to chromosome 12, which overlapped with the Iddm30 locus previously shown to control T1D susceptibility and the severity of insulitis (2). As expected from the phenotype of the parental strains, the BBBDP allele at Iddm30 imparts a significant increase in pancreatic Ccl11 transcription levels compared with the ACI allele. Because at the peak of linkage levels of Ccl11 transcripts are similar in F<sub>2</sub> animals that are heterozygotes and ACI homozygotes, the inheritance of this trait appears to be recessive at this locus. Strikingly, however, whereas results of the linkage analysis establish the genetic control of this trait, they also show that, at the peak of linkage, the BBBDP genotype that is associated with pancreatic overexpression of Ccl11 confers recessive resistance to T1D and insulitis (2). Although at this stage it is premature to consider that Iddm30 and the chromosome 12 locus controlling the levels of Ccl11 expression are allelic, this observation suggests that pancreatic overexpression of Ccl11 is protective against T1D.

Linkage of pancreatic Ccl11 expression to chromosome 12 (LOD = 3.7) is relatively weaker than that of T1D (LOD = 3.8) and insulitis (LOD = 6.4). However, it is important to note that Ccl11 expression was only assessed in 117 F<sub>2</sub> rats, a number considerably smaller than that (n = 574) used for the mapping of the two other traits (2). Furthermore, the proportion of the Ccl11 trait variance accounted for by this locus, 12.2%, is much higher than that previously reported for T1D (3.1%) and insulitis (5.1%), reflecting the strong effect of this locus on pancreatic expression of Ccl11 (2). Although one could argue that the linkages of Ccl11 expression, T1D, and insulitis to chromosome 12 are not perfectly overlapping, one should consider two explanations that may have led to a less accurate mapping of the Ccl11 expression trait. One is the relatively low number of animals assessed for Ccl11 expression, and the second is the way these animals were selected. Specifically, to avoid the confounding effects of age on pancreatic expression of Ccl11, we restricted the assessment of this trait to F<sub>2</sub> animals that had remained T1D free until the end of the follow-up period. This selection had two consequences. First, this nondia-
betic cohort \((n = 117)\) was enriched in the proportion of \(F_2\) animals that were homozygous for the BBDP T1D resistance allele at the peak of \(Iddm30\) (34.8\%) when compared with the whole cohort of \(F_2\) rats \((n = 574)\), in which the expected Mendelian proportion of BBDP homozygous animals was observed (25.3\%). Second, it was most likely that by selecting for nonobese \(F_2\) animals, we enriched for high expressers of pancreatic \(Ccl11\) among those that were heterozygous and ACI homozygous at the peak of linkage on chromosome 12, thereby minimizing the differential \(Ccl11\) expression between the two genotypes.

The one LOD interval surrounding the peak of linkage of pancreatic \(Ccl11\) (Eotaxin) expression on chromosome 12 is large, 36.4\,cM or 20.5\,Mb, and therefore contains many candidate genes, including Eotaxin-2 (\(Ccl24\)) and Eotaxin-3 (\(Ccl26\)), that map in close proximity to the peak of linkage (http://www.tdlabase.org/) and share the same receptor Ccr3 with \(Ccl11\). Given the genomic location of \(Ccl24\) and \(Ccl26\) and the evidence that the regulation of their expression is coordinated with that of \(Ccl11\) in allergic and other Th2-mediated immune responses (32, 34, 45), we sequenced the coding regions of these two genes and assessed their transcript levels in the pancreas of BBDDP and ACI.1u.1lyp parental strains. Of note, \(Ccl11\) itself is located on chromosome 10, and it was therefore plausible that a chromosome 12 genetic polymorphism in our cross would differentially regulate the expression of \(Ccl11\) in trans and that of the other eotaxins in cis. However, whereas we detected a nonsynonymous single-nucleotide polymorphism in exon 1 of \(Ccl24\) at position 19 in which a G in BBDDP to A in ACI substitution resulted in a conservative V to I substitution at aa 7, there was no evidence for differential expression of \(Ccl24\) and 26 in the pancreas of the two parental strains (data not shown).

Constitutive expression of various cytokines by pancreatic islets has been observed in rodents and humans (25, 26), but its physiological significance remains unknown. However, it has been demonstrated in vitro that interaction of \(\beta\) cells with extracellular matrix proteins results in the induction of chemokine expression in these cells through NF-\(\kappa\)B activation (27, 49). This activation of NF-\(\kappa\)B is sustained through an IL-1- mediated autocrine loop and appears to be important for optimal insulin secretion and \(\beta\) cell survival (27, 49). In this context, analysis of the promoter region of \(Ccl11\) reveals the presence of a consensus NF-\(\kappa\)B binding site (−68 to −59 bp), and NF-\(\kappa\)B and STAT6 activation have been shown to induce \(Ccl11\) transcription in airway epithelial cells (50).

It is therefore possible that the constitutive activation of NF-\(\kappa\)B required for \(\beta\) cell function and survival results in the coordinated expression of cytokines by \(\beta\) cells. The question follows as to whether dysregulated expression of these inflammatory mediators in \(\beta\) cells could have an impact on antipancreatic autoimmunity. The functional analysis of the T cells infiltrating the pancreatic islets of \(Iddm30\) congenic inbred BBDDP animals suggests that it does.

To confirm the linkage of T1D and pancreatic expression of \(Ccell1\) to chromosome 12, we developed \(Iddm30\) congenic BBDDP rats through the introgression of an ACI- derived, ~36-Mb interval of chromosome 12 into the BBDDP background. The significantly higher susceptibility of the resulting BB-ACI- \(Iddm30\) animals to T1D compared with BBDDP rats confirmed the influence of this locus on disease pathogenesis. Likewise, the observation that the levels of \(Ccell1\) transcripts in the pancreas of adult BB-ACI- \(Iddm30\) animals were similar to those of age-matched ACI.1u.1lyp but significantly lower than those of BBDDP animals confirmed the influence of the \(Iddm30\) locus on the regulation of this trait.

The critical role of \(Ccl11\) in driving the recruitment of Ccr3- expressing Th2 lymphocytes, mast cells, and eosinophils to the site of atopic and other Th2-mediated immune responses is well characterized (32, 34, 45, 51). Through this recruitment and the cytokines released by the attracted cells, \(Ccl11\) contributes to the skewing of Th differentiation away from Th1 to Th2 (34). T1D is a prototypical Th1-driven autoimmune disease (38–40), and it has been shown that genetically determined or therapeutically induced shifts of the intraislet Th1/Th2 balance toward Th2 can prevent the disease in rodent models (19, 38, 43, 52). The differential expression of \(Ccl11\) in the pancreas of T1D-resistant and T1D-prone rats led us to determine whether overexpression of this cytokine was associated with evidence of a Th2 skewing among the CD4\(^+\) T cells present in inflamed islets. The proportion of intraislet T cells and, among those, that of CD4\(^+\) cells synthesizing IFN-\(\gamma\) upon activation was similar between \(Iddm30\) congenic BBDP animals. In contrast, there was an increased proportion of IL-4- synthesizing CD4\(^+\) T cells in the islets of animals homozygous for the BBDDP allele at the \(Iddm30\) locus shown in this work to be associated with high levels of pancreatic \(Ccl11\) expression. Consistent with this Th2 shift among intraislet CD4\(^+\) T cells, we also detected a 2-fold increase in \(Gata3\) transcript levels in the pancreas of nonobese BBDDP rats compared with their age-matched BB-ACI- \(Iddm30\) counterparts. Of note, we did not observe a differential recruitment of mast cells known to express Ccr3 to the pancreatic islets. In fact, we could hardly detect mast cells in the insulitic lesions of our diabetic and nonobese \(Iddm30\) congenic BBDDP animals. Taken together, results presented in this study suggest that the BBDDP allele of \(Iddm30\) confers resistance to destructive insulitis and diabetes and preferential recruitment of Th2 CD4\(^+\) T cells over Th1 cells as a consequence of increased \(Ccl11\) synthesis by pancreatic \(\beta\) cells.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplementary Figure 1.
Supplemental Material

Supplemental Figure Legend

Supplementary Figure 1. Physical mapping of the ACI.1u.lyp-derived chromosome 12 congeneric interval in the inbred BB.AC1-Iddm30 strain. Physical locations (Mb) of relevant microsatellite markers are indicated on the left of the chromosome. The introgressed ACI chromosome 12 interval (<35.99 Mb) is shown in black, the BBDP genetic background in white, and the regions of unknown genotype are striped. The 1-LOD interval of Iddm30 is shown by the arrowed line.