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

Gary Y. C. Chao, Robert H. Wallis, Leili Marandi, Terri Ning, Janice Sarmiento, Andrew D. Paterson and Philippe Poussier

*J Immunol* 2014; 192:3645-3653; Prepublished online 19 March 2014;
doi: 10.4049/jimmunol.1302383
http://www.jimmunol.org/content/192/8/3645

**Supplementary Material**  [http://www.jimmunol.org/content/suppl/2014/03/19/jimmunol.1302383.DCSupplemental](http://www.jimmunol.org/content/suppl/2014/03/19/jimmunol.1302383.DCSupplemental)

**References** This article cites 51 articles, 33 of which you can access for free at: [http://www.jimmunol.org/content/192/8/3645.full#ref-list-1](http://www.jimmunol.org/content/192/8/3645.full#ref-list-1)

**Subscription** Information about subscribing to *The Journal of Immunology* is online at: [http://jimmunol.org/subscription](http://jimmunol.org/subscription)

**Permissions** Submit copyright permission requests at: [http://www.aai.org/About/Publications/JI/copyright.html](http://www.aai.org/About/Publications/JI/copyright.html)

**Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at: [http://jimmunol.org/alerts](http://jimmunol.org/alerts)
Iddm30 Controls Pancreatic Expression of Ccl11 (Eotaxin) and the Th1/Th2 Balance within the Insulitic Lesions

Gary Y. C. Chao,* Robert H. Wallis,* Leili Marandi,* Terri Ning,* Janice Sarmiento,*† Andrew D. Paterson,‡ and Philippe Poussier*†§

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 diabetes 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-independent pancreatic upregulation of Ccl11 in BBDR rats when compared with T1D-resistant ACI.1u.lyp 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 insulitic 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 inflammation 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 α haplotype (Iddm1) on chromosome 20 (3, 8, 9) and a recessive null mutation of GTPase, 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 BBBD rat, the chromosomal intervals of these loci were large, thus making the search for the causal genes within 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 BBBD 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 BBBD/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 DRlyp/lyp 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). Cell11 is one of the three members of the eotaxin family of C-C chemokines that are potent chemoattractants for CCr3-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 BBDP rats has been previously reported (35–37), the observation that Cell11 is upregulated in the target organ of this diabeticogenic process remains puzzling (24, 37), particularly because diabetes in the BBDD rat is a Th1-mediated autoimmune disease (38–40). Notwithstanding, Hassner’s group (37) examined the role of pancreatic Cell11 expression in T1D pathogenesis in DR<sup>hyp/typ</sup> 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 Cell11 may be pathogenic (37).

In this study, we have tested the hypothesis that pancreatic overexpression of Cell11 is genetically determined and contributes to the regulation of the diabeticogenic process in the BBDD rat. We observed a differential pancreatic expression of Cell11 between T1D-susceptible BBDD and T1D-resistant ACI.1u.lyp rat strains. Specifically, Cell11 transcript levels were upregulated in a time-dependent fashion in the BBDD rat pancreas, and this upregulation was confirmed to be Iddm30 independent. Through linkage analysis of a cross-intercross of BBDD 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 BBDD strain congenic for the chromosome 12 interval that controls T1D, insulitis, and Cell11 expression in pancreatic islets. Importantly, our data show that whereas the BBDD allele at Iddm30 is associated with the expected higher levels of pancreatic Cell11, this allele confers resistance to T1D. Further analysis of islet-infiltrating T cells in Iddm30 congenic BBDD animals revealed an increased proportion of IL-4-synthesizing CD<sup>+</sup> T cells in rats homozygous for the BBDD allele. Taken together, our results strongly suggest that the elevated recruitment of these Th2 cells was driven by the pancreatic overexpression of Cell11 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 BBDD 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 BBDD 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 BB.ACI-Iddm2 rats (BB.NonLyp) congenic for wild-type Gimap5 have been previously described (15). The inbred ACI.1u and ACI.1u.lyp strains were derived from the August Copenhagen Irish (ACI)/Hsd rats (Harlan Sprague Dawley, Indianapolis, IN) through introgression of the BBDD RT1<sup>v</sup> (Iddm01) locus alone or in combination with the BBDD Gimap5 (Lyp) locus (Iddm30) (2). To generate the (BBDD × ACI.1u)F<sub>1</sub> lymphocyte (BBDD males were first crossed with ACI.1u.lyp females to generate F<sub>1</sub> progeny 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 BBDD rats

For generation of the congenic inbred BBDD.ACI-Iddm30 (BB.ACI-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 F<sub>2</sub> linkage analysis (2) was introgressed into the BBDD background. Specifically, BBDD and ACI.1u.lyp rats were first intercrossed, and this was followed by 10 successive, marker-assisted backcrosses to the BBDD 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 F<sub>2</sub> 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 selected for further analysis. Further refinement of linkage was obtained through genotyping of additional markers flanking these peaks of linkage, following 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 iQ<sub>SYBR</sub> Green supermix with ROX (Bio-Rad, Hercules, CA), β-actin primers (forward, 5′-CTT TAA GGC CCA TCC TTT-3′; reverse, 5′-ACC AGC GAG CTA CAG GGA CAA-3′; Sigma Genosys) as quantification standard, and Cell11 primers (forward, 5′-CAG TCT TCC ACA GCA CCT CT-3′; reverse, 5′-CTG GTC ATG GTA AAG CAG 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 ΔΔ Ct method to calculate the relative transcript copy number of Cell11 to β-actin. Unless otherwise stated, 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 Cell11 and pancreatic hormones used goat polyclonal anti-Cell11 (R&D Systems, Minneapolis, MN) and mouse monoclonal anti-insulin or anti-glucagon Abs (Sigma-Aldrich), and was performed as described previously (37). In brief, freshly excised pancreata 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 on t-lens–coated slides and mounted onto t-lens–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 Abs, Texas Red–conjugated donkey anti-goat IgG and FITC-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories), for 1 h at room temperature. Slides were washed, dried, and mounted using fluorescence mounting medium (Dako). Slide analysis was done on a Zeiss Axiosvert 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 dissected 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 dithiozone (Sigma-Aldrich) in 10 mM HEPES-buffered PBS solution (42). Collected islets were then lysed in 1% Nonidet P-40 (43) in 0.01% w/v dithizone (Sigma-Aldrich) in 10 mM HEPES-buffered PBS solution (42). Equal amounts of islet-derived protein extract and the total protein concentration was determined by bicinchoninic acid assay (Life Technologies). Islets from 90-d-old BBDP rats were incubated with protein A–Sepharose beads (Sigma-Aldrich) pre-assay (Life Technologies). Equal amounts of islet-derived protein extract were incubated with protein A–Sepharose beads (Sigma-Aldrich) pre-coupled with rabbit polyclonal anti-rat Ccl11 Ab (Peprotech, Rocky Hill, NJ) for immunoprecipitation (IP) at 4°C overnight. To determine whether pancreatic islets of diabetes-prone (DR) and diabetes-resistant (WF) strains grown progressively larger over time. Having demonstrated that pancreatic 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 BBDP 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 was 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 BBDP 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 BBDP 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 BBDP 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 BBDP donors as early as 30 d of age (p = 0.01), 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 BBDP 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 BBDP × ACI.1u.lypF2 animals was initiated when the development of this cohort was well underway. F2 animals (n = 574) were followed prospectively until the onset of TID (n = 127) or up to 165 d for nondiabetic animals. Of the 447 animals that remained...
diabetes free, we assessed the pancreatic levels of \( \text{Ccl11} \) transcripts in the last 117 rats followed. There were two reasons for excluding diabetic rats from our analysis. First, they would not be age matched to nondiabetic rats, and this age difference would be a confounding factor because our data showed that age has a profound impact on the pancreatic levels of \( \text{Ccl11} \) transcripts (Fig. 2B). Second, we reasoned that the destruction of \( \beta \) cells by the diabetogenic process would remove the main source of \( \text{Ccl11} \) expression in the pancreas.

The (BBDP \( \times \) ACI.1u.lyp)\( F_2 \) animals assessed for pancreatic \( \text{Ccl11} \) transcript levels were genotyped across the genome using 229 microsatellite markers at an average of one marker every 12.5 Mb, as previously described (2). This was followed by linkage analysis to determine which chromosomal region(s) controls this quantitative trait. Only one region, on chromosome 12, showed significant linkage. The peak of linkage (LOD = 3.65) is located at marker D12Rat51, and the 1-LOD interval of linkage spans 36.4 cM between markers D12Rat28 and D12Rat52 (Fig. 3A). This locus alone accounts for 12.2% of the trait variance observed in this cross by ANOVA. As expected based on parental phenotypes, when the \( \text{Ccl11} \) transcript levels of the 117 \( F_2 \) rats were compared based on the BBDP or ACI genotype of these animals at the peak of linkage (Fig. 3B), those that were BBDP homozygous had significantly higher levels than ACI homozygotes (\( p = 0.0006 \)) and heterozygotes (\( p = 0.0007 \)). In contrast, there was no significant difference in \( \text{Ccl11} \) 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 \( \text{Ccl11} \) overlaps with the \( \text{Iddm30} \) locus that was found to control the severity of insulitis and T1D susceptibility in the whole cohort of 574 (BBDP \( \times \) ACI.1u.lyp)\( F_2 \) animals (Fig. 3A) (2). The 117 nondiabetic animals assessed for transcript levels of \( \text{Ccl11} \) in the pancreas were derived from the last 167 members of the whole (BBDP \( \times \) ACI.1u.lyp)\( F_2 \) cohort, of which 45 (26.9%) became diabetic. The original report that \( \text{Ccl11} \), 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 \( \text{Ccl11} \) pancreatic transcripts in the (BBDP \( \times \) ACI.1u.lyp)\( F_2 \) cohort, the same genotype would also confer a high risk for T1D. As shown in Fig. 3C, this was not the case. Specifically, \( F_2 \) 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 \( \text{Ccl11} \) 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 \( \text{Ccl11} \) expression in the pancreas is under the control of a chromosome 12 locus that overlaps to a large extent with \( \text{Iddm30} \), and the high levels of pancreatic \( \text{Ccl11} \) expression are associated with protection from T1D.

The phenotypic analysis of BBDP rats congenic for \( \text{Iddm30} \) confirms linkage of T1D and pancreatic \( \text{Ccl11} \) expression to this locus

To confirm the results of linkage analyses, an inbred BBDP \( \text{Iddm30} \) congenic line named BB.ACI-\( \text{Iddm30} \) was developed.
transcripts in their pancreas. This parameter was assessed in Iddm30 resistance to T1D. Kaplan–Meier survival analysis. These results confirm that homozygosity for the BBDP allele at Iddm30 confers significant resistance to T1D and elevated levels of Ccl11 expression in the pancreas.

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 BBDP 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 BBDP 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 BBDP (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 BBDP 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 BBDP 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 BBDP 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 homozygosity for the BBDP allele at Iddm30 confers resistance to T1D and is associated with an increase in both pancreatic expression of Ccl11 and differentiation of insulinic 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 BBDP 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 BBDP 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 BBDP animals (75%, n = 36; p = 0.045 by one-tailed χ² test). Of note, the age of disease onset was not significantly different between the two Iddm30 congenic strains (78 ± 9.9 d in BBDP versus 82 ± 8.9 d in BB.ACI-Iddm30, mean ± 1 SD; p = 0.4 by Kaplan–Meier survival analysis). These results confirm that homozygosity for the BBDP 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 BBDP Iddm30 congenic animals. As illustrated in Fig. 4B, the levels of Ccl11 transcription were significantly higher in the pancreas of BBDP 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 BBDP 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 BBDP 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 BBDP; 564.5 ± 513.9/10^3 β-actin copies, n = 9 in BB.ACI-Iddm30 rats, p = 0.012 versus BBDP). Thus, the analysis of Iddm30 congenic inbred animals confirms the linkage results whereby homozygosity for the BBDP 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 BBDP 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 BBDP, ACI.1u.lyp, and their respective Iddm2 (lyp) congenic counterparts, BB.NonLyp and ACI.1u 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 BBDP 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-

FIGURE 3. Pancreatic transcript levels of Ccl11 are linked to a chromosome 12 region that also controls T1D and severity of insulitis in a cohort of (BBDP × ACI.1u.lyp)F2 animals. (A) Linkage plots for T1D (solid line), insulitis (dashed line), and pancreatic Ccl11 transcript levels (dotted line) on chromosome 12. The peaks of linkage for T1D (LOD = 3.76) and insulitis (LOD = 6.44) are located at the same marker D12Rat28 that is only 2.96 cM away from the peak of linkage for pancreatic transcript levels of Ccl11 (LOD = 3.65) at marker D12Rat51. Chromosomal locations are indicated in cM along the x-axis. (B and C) Influence of the genotype (ACI allele = A; BBDP allele = B) of F2 animals at marker D12Rat51 on pancreatic transcript levels of Ccl11 and T1D, respectively. (B) 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. (C) Cumulative survival of T1D-free F2 animals according to their genotype at D12Rat51 (statistical analyses were performed by Kaplan–Meier survival analysis, and significant p values are indicated).

FIGURE 4. Phenotypic analysis of inbred Iddm30 congenic BBDP animals confirms linkage of T1D and pancreatic Ccl11 expression to rat chromosome 12. (A) Cumulative survival of T1D-free BBDP and BB.ACI-Iddm30 animals. Survival of BBDP animals (25%, n = 36) is shown by the solid line, whereas survival of the BB.ACI-Iddm30 animals (8%, n = 25) is shown by the dashed line (statistical analysis of significant difference in survival was performed by χ2 test). There was no difference in age of onset between the two strains (median of 83 and 79 d for BBDP and BB.ACI-Iddm30, respectively). (B) Comparison of pancreatic transcript levels of Ccl11 in 90-d-old BBDP, ACI.1u.lyp, and BB.ACI-Iddm30 animals as assessed by qRT-PCR analysis. (C) Time-dependent upregulation of pancreatic Ccl11 transcript levels among these three strains. There was no significant difference in Ccl11 transcript levels between age-matched ACI.1u.lyp and BB.ACI-Iddm30 rats, whereas levels in BBDP were significantly higher than in the two other strains at each age examined. Bars indicate mean transcript levels ± SEM; statistical analyses were performed by t test, and significant p values (p < 0.05) are indicated unless no significance (n.s.) in difference was found.
animals and assessed for proportion of CD4 + T cells that synthesized IL-4.

Analyses comparing the parental strain were performed by multicolor immunofluorescence and FACS analysis. Individual values of each sample are shown, and bars represent mean ± SEM. Statistical analyses comparing Iddm30 congenic BBBDP animals with the BBBDP parental strain were performed by t test, and significant p values (p < 0.05) are indicated.

ululated in these same animals (48). However, to the best of our knowledge, there is no evidence that these β cell abnormalities are genetically determined nor can one rule out the possibility that some of them are the consequence of the autoimmune process. In contrast, the time-dependent expression of Ccl11 in the β cells of diabetes-prone DR rats reported by Hessner et al. (24) appeared genetically determined and unlikely to result from islet inflammation. Furthermore, the same group of investigators showed that increased pancreatic expression of the chemokine Ccl11 in DRbbbp animals was associated with an elevated number of activated mast cells in pancreatic lymph nodes that may have contributed to the acceleration of the diabetogenic process in these animals (37).

In the current study, we tested the hypothesis that pancreatic overexpression of Ccl11 is genetically determined and contributes to the regulation of the diabetogenic process in the BBBDP rat. Through genetic linkage analysis and the development of a novel congenic inbred line of BBBDP rats, we demonstrate that overexpression of Ccl11 is under the control of a region of rat chromosome 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 DRbbbp 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.1yp, 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.1yp 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.1yp 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, nondiabetic (BBBDP × ACI.1u.1yp)F2 animals showed significant linkage of pancreatic expression of Ccl11 only to a chromosome 12 region, 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 F2 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 F2 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 F2 animals that had remained T1D free until the end of the follow-up period. This selection had two consequences. First, this nondia-
otic cohort \((n = 117)\) was enriched in the proportion of \(F_2\) animals that were homozygous for the BBBDP 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 BBBDP homozygous animals was observed \((25.3\%)\). Second, it was most likely that by selecting for nondiabetic \(F_2\) 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 (Ccl124) and Eotaxin-3 (Ccl26), that map in close proximity to the peak of linkage \((http://www.t1dbase.org/)\) 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 BBBDP and ACI.1u.Iyp 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 two 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 BBBDP 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-activated 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 consensual 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 BBBDP animals suggests that it does.

To confirm the linkage of T1D and pancreatic expression of Ccl11 to chromosome 12, we developed Iddm30 congenic BBBDP rats through the introgression of an ACI-derived, ~36-Mb interval of chromosome 12 into the BBBDP background. The significantly higher susceptibility of the resulting BB-ACI-Iddm30 animals to T1D compared with BBBDP rats confirmed the influence of this locus on disease pathogenesis. Likewise, the observation that the levels of Ccl11 transcripts in the pancreas of adult BB-ACI-Iddm30 animals were similar to those of age-matched ACI.1u.Iyp but significantly lower than those of BBBDP 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 prototypic Th1-driven autoimmune disease \((38\text{"}40)\), 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 BBBDP animals. In contrast, there was an increased proportion of IL-4–synthesizing CD4\(^+\) T cells in the islets of animals homozygous for the BBBDP 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 nondiabetic BBBDP 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 nondiabetic Iddm30 congenic BBBDP animals. Taken together, results presented in this study suggest that the BBBDP 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.

Acknowledgments

We thank Dr. Martin J. Hessner for providing the protocols for immunofluorescent staining of pancreatic sections, Kirishanthy Kathirakambathamy from the Sunnybrook Antibody Core Facility, Gisele Knowles from the Sunnybrook Flow Cytometry Core Facility, Catherine Gibson from Sunnybrook Comparative Research for Excellent Animal Care, and Dr. Jayne Danska for a critical review of this manuscript.

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 congenic interval in the inbred BB.ACI-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.