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Full-Text

Title: Pathogenetic role of an autoimmune susceptibility locus derived from MRL/MpJ strain chromosome 1 in chronic pancreas inflammation

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Running head: Autoimmune-mediated murine pancreatitis

Abstract

We examined the role of *Mag*, an autoimmune susceptibility locus encoded by the telomeric region of MRL/MpJ mouse chromosome 1, in the pathogenesis of autoimmune exocrinopathy. At 9–12 months of age, strain-specific differences were observed in the pancreas. B- and T-cell-containing periductal/perivascular cell infiltrations in the pancreases of MRL/MpJ and B6.MRLc1 congenic C57BL/6-background *Mag*-carrying strains were more severe than that of C57BL/6. Pancreatic periductal/perivascular cell infiltration was observed frequently in A/J, AKR/N, B6.MRLc1, C57BL/6, and MRL/MpJ, moderately in DBA/1 and DBA/2, and rarely in BALB/c and C3H/He strains. Females tended to have greater pancreatic periductal/perivascular cell infiltration than males. C57BL/6 mice possessed defined borders between cell infiltrations and acini, but borders were indistinct in MRL/MpJ and B6.MRLc1 mice. We attributed this to the invasion of inflammatory cells between each acinus and the disruption of acinar cells around cell infiltrations in the latter strains. No strain-specific differences were observed in the appearance of fibrotic lesions and high endothelial venules in the cell infiltrates. The levels of serum anti-dsDNA antibody and amylase, and mRNA expression of tumor necrosis factor-α and Fc gamma receptor III (both encoded on *Mag*) in the pancreases were elevated in MRL/MpJ and B6.MRLc1 strain mice relative to C57BL/6. These results emphasized

the crucial roles of *Mag* in the molecular and genetic pathogenesis of autoimmune-mediated pancreatitis.

Keywords

Telomeric region of chromosome 1, MRL/MpJ mouse, inflammatory exocrinopathy, pancreas

Introduction

Inflammation of exocrine glands is caused by bacterial or viral infection, allergy, and autoimmune diseases.[1-3] Two autoimmune-mediated exocrinopathies, Sjögren syndrome (SS) and autoimmune pancreatitis (AIP), have been well described. In the United States, primary SS affects an estimated 0.4−3.1 million individuals.[4] SS is characterized by lymphocytic infiltration in the labial salivary or lacrimal glands, leading to the hyposecretion of saliva or lacrimal fluid.[4,5] In Japan, an estimated 6,700−26,000 patients are affected by AIP.[6] According to its histopathological features, AIP is classified into two subtypes. Briefly, Type 1 AIP, lymphoplasmacytic sclerotic pancreatitis, is characterized by the abundant infiltration of IgG4-positive plasma cells and lymphocytes, peculiar storiform or swirling fibrosis, and perivenular infiltration that often leads to obliterative phlebitis. Type 2 AIP, idiopathic duct-centric pancreatitis, is characterized by the infiltration of granulocytes that often leads to the destruction and obliteration of pancreatic ducts. In Type 2 AIP, there is no involvement of IgG4.[3]

Autoimmune-mediated exocrinopathy has been investigated in NOD, MRL/MpJ-*lpr/lpr*, and NZB/NZWF1 model mice. In addition, previous studies have demonstrated that BXSB/MpJ mice develop an autoimmune dacryoadenitis characterized by B-cell-predominant infiltrates.[4] MRL/MpJ mice, including their mutant strain *lpr/lpr*,

spontaneously develop inflammation in lacrimal glands, salivary glands, and the pancreas.[7,8] Because C57BL/6 and C3H/He mice carrying the *lpr* allele do not show an autoimmune phenotype,[9] the autoimmune phenotypes associated with the MRL/MpJ strain must depend on the genetic background in addition to the presence of the *lpr* allele.[10] These results emphasize the crucial impact of genetic background on the development of autoimmune diseases. In fact, several autoimmune disease-associated genetic loci have been identified in murine models. *Swrl-1* (autoantibody), *Sle1* (autoantibody, splenomegaly), and *Aec2* (autoimmune exocrinopathy) are reportedly the susceptibility loci in SWR, NZW, and NOD mice, respectively.[11,12] B6.MRL(*D1Mit202–403*) (B6.MRLc1) mice, a C57BL/6-background congenic mouse carrying the telomeric region of MRL/MpJ-derived chromosome 1 (*D1Mit202–403*, 67.97–81.63 cM, Mouse Genome Informatics, http:www.informatics.jax.org/; 1:157,040,949–175,641,372, Ensembl, http://asia.ensembl.org/index.html), were previously generated.[13] Although this MRL/MpJ-derived congenic locus, MRL autoimmune glomerulonephritis (*Mag*), contributed to the development of the high serum level of anti-double strand DNA (dsDNA) antibody, splenomegaly, and the autoimmune glomerulonephritis,[14] the pathological roles of this locus in exocrine glands has not been evaluated.

Mag encoded approximately 200 genes (Ensembl), and it has been suggested that the

telomeric region of human chromosome 1 and *Mag* both encode several critical immune-associated genes including Fas ligand (*Fasl*), selectins (*Sel*), and Fc gamma receptor (*Fcgr*) family members (UCSC Genome Bioinformatics; http://genome.ucsc.edu/), indicating shared synteny in humans and mice.[9,12,15] In SS patients, Fas is expressed in epithelial cells and mononuclear cells in the salivary glands, and FasL expression is upregulated specifically in infiltrating T-cells, suggesting a pathological correlation between SS and Fas/FasL-mediated apoptosis.[16] Furthermore, serum expression of E-, L-, and P-selectins participate in cell infiltration changes in acute pancreatitis patients.[17] Altered affinity or function of Fc gamma receptors (FcγRs, encoded by *Fcgr* genes) have been reported as a risk factor for human autoimmune diseases,[18] and it is suggested that elevated ratio of active-type FcγRIII to inhibitory-type FcγRIIB is considered as a crucial cause of the autoimmune-prone phenotype in the B6.MRLc1 mouse strain.[14] Thus, the telomeric region of murine chromosome 1, *Mag*, is a hotspot regulating autoimmune disease susceptibility.

In the present study, in order to reveal relationship between *Mag* and inflammatory exocrinopathy, B6.MRLc1 mice were analyzed and compared to C57BL/6 and MRL/MpJ strains. We found that the B6.MRLc1 mice developed chronic pancreatitis and possessed elevated serum pancreatitis markers and mRNA expression of inflammatory mediators

and autoimmune-associated genes encoded on *Mag*. This finding indicates that B6.MRLc1 could serve as a murine model of autoimmune-mediated pancreatitis and emphasized the crucial roles of *Mag* in the molecular and genetic pathogenesis of this disease.

Materials and Methods

Animals and sample preparation

We adhered to *the Guide for the Care and Use of Laboratory Animals of Hokkaido University, Graduate School of Veterinary Medicine* (approved by the Association for Assessment and Accreditation of Laboratory Animal Care International). B6.MRLc1 mice were C57BL/6-type homozygous in *D1Mit64–200* (3.67–63.84 cM, 1:12,846,696–150,318,120) and *D1Mit361* (89.95 cM, 1:186,261,829-186,261,999), and MRL/MpJ-type homozygous in *D1Mit202–403* (67.97–81.63 cM, 1:157,040,949−175,641,372). B6.MRLc1 were also C57BL/6-type homozygous in chromosomes 2−19, X, and Y.[13] There are several immune-associated genes in *D1Mit202-403* including *Fasl*, selectin endothelial cell (*Sele*), selectin lymphocyte (*Sell*), selectin platelet (*Selp*), *Fcgr2b*, and *Fcgr3*.

Both sexes of B6.MRLc1, over 6 months of age, were bred in the laboratory. A/J, AKR/N, BALB/c, C3H/He, C57BL/6, DBA/1, DBA/2, and MRL/MpJ male and female mice, aged 5−7 months, were purchased from Japan SLC, Inc. (Hamamatsu, Japan). The mice were maintained under specific pathogen-free conditions. All mice were sacrificed by exsanguination from the carotid arteries under deep anesthesia (Avertin; 2,2,2-tribromoethanol dissolved in 2-methyl-2-butanol, 2,400 mg/kg, administered intraperitoneally), and the salivary glands, pancreas, and serum were immediately collected.

The salivary glands and pancreas samples for histology were fixed in 4% paraformaldehyde (PFA) at 4°C overnight, and a portion of each pancreas was frozen in OCT. compound (Sakura Finetek, Tokyo, Japan).

Histology and histometrical analysis

Paraffin sections (3-µm-thick) were prepared and stained with hematoxylin-eosin (HE). The area of inflammatory cell infiltration was measured in salivary gland and pancreas cross-sections visualized using a BZ-9000 microscope (KEYENCE, Osaka, Japan). The ratio of cell infiltration area to the total cross-section area was expressed as percentage. The frequency of pancreatic cell infiltration was also investigated in A/J, AKR/N, BALB/c, B6.MRLc1, C3H/He, C57BL/6, DBA/1, DBA/2, and MRL/MpJ mice. Briefly, pancreases from four mice/strain were investigated and scored from – to ++. −: no cell infiltration; +: cell infiltrates were observed in one or two mice; ++: cell infiltrates were observed in three or four mice.

Blood biochemistry

The levels of serum amylase and lipase were measured using the Fuji Dri-Chem analyzer (Fujifilm Medical Co. Ltd., Japan) at the Japan Food Research Laboratories

(Hokkaido, Japan). The level of serum anti-dsDNA antibody was measured using an ELISA kit (Cat No. 5110; Alpha Diagnostics International Inc., Texas, USA). The serum blood glucose was measured using the MS-GR-102 blood glucose meter (Terumo, Tokyo, Japan).

Immunohistochemistry and histometrical analysis

To detect the T-cell marker CD3, the B-cell marker B220, and the lymphatic vessel marker lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1) by immunohistochemistry, paraffin sections were deparaffinized and incubated in citrate buffer (pH 6.0) for 20 min at 105°C for antigen retrieval. For peripheral node addressin (PNAd, ligand of L-selectin), alpha smooth muscle actin (αSMA), and FcγRIII (encoded by *Fcgr3*), 5-μm-thick frozen sections were prepared, fixed using ice-cold 4% PFA, and completely dried. The slides were next soaked in methanol containing 0.3% H_2O_2 for 30 min at room temperature to remove endogenous peroxidases. After washing with 0.01 M phosphate-buffered saline (PBS), sections were incubated with 0.25% casein/PBS for 60 min at room temperature and then with rabbit IgG antibodies for CD3 (1:150, Nichirei, Tokyo, Japan), rat IgG antibodies for B220 (1:1000, Cedarlane, Ontario, Canada), rabbit IgG antibodies for LYVE1 (1:500, Adipogen San Diego, CA USA), rat IgM antibodies for PNAd (1:500, Biolegend, London, UK), or rabbit IgG antibodies for αSMA (1:3000, Abcam,

154 Cambridge, UK) overnight at 4°C. After washing three times in PBS, sections were
155 incubated with biotin-conjugated mouse anti-rat IgM antibodies (1:250, Biolegend, London,
156 UK) for PNAd, biotin-conjugated goat anti-rabbit IgG antibodies (SABPO kit, Nichirei,
157 Tokyo, Japan) for CD3 and αSMA, or goat anti-rat IgG antibodies (Caltag-Medsystems
158 Limited, Buckingham, UK) for B220 for 30 min at room temperature. Sections were washed
159 again and incubated with streptavidin-biotin complex (SABPO kit, Nichirei) for 30 min. The
160 positive reactions were then visualized using 3,3′-diaminobenzidine
161 tetrahydrochloride-H_2O_2 solution. Finally, the sections were lightly counterstained with
162 hematoxylin.
163
164 *Terminal deoxynucleotidyl transferase dUTP nick end labeling analysis*
165 *Terminal deoxynucleotidyl transferase dUTP nick end labeling* (TUNEL) staining was
166 performed on the frozen pancreas sections using the Apoptosis in situ Detection Kit Wako
167 (Wako Pure Chemical Industries, Ltd. Osaka, Japan) following the manufacturer's protocol.
168 Briefly, 5-μm-thick frozen sections were prepared, fixed using ice-cold 4% PFA, and
169 completely dried. The sections were permeabilized with 0.1% sodium citrate and 0.1% Triton
170 X on ice for 2 min and washed with PBS. To label the 3′-terminals of DNA, the sections
171 were incubated with TdT reaction solution at 37°C for 10 min. After washing three times in

PBS, endogenous peroxidases were inactivated using 3% H_2O_2 at room temperature for 5 min and then washed three times in PBS. The sections were next labeled by peroxidase-conjugated antibody. After washing three times in PBS, the sections were incubated with 3,3′ -diaminobenzidine tetrahydrochloride-H_2O_2 solution. Finally, the sections were lightly counterstained with hematoxylin.

Quantitative reverse transcription polymerase chain reaction

To examine mRNA expression, total pancreatic was isolated using the TRIzol Reagent (Ambion, Texas, USA) following the manufacturer's protocol. DNase-treated total RNA was reverse-transcribed to cDNA using the GoScript™ Reverse Transcription System (Promega, Wisconsin, USA). Each cDNA, adjusted to 50 μg/ml, was used as template in quantitative polymerase chain reaction (qPCR) using the Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent, California, USA). The primer pair sequences are shown in Table 1.

Statistical analysis

Results were expressed as the mean ± standard error (SE), and the non-parametric Mann-Whitney *U* test was performed to compare between C57BL/6 and the other strains ($P < 0.05$).

Results

Histopathology of mouse exocrine glands

To determine the pathological roles of *Mag*, we compared the histological features of exocrine glands including parotid glands, sublingual glands, and pancreases from C57BL/6, B6.MRLc1, and MRL/MpJ female mice at 9–12 months of age (Figure 1). In the examined exocrine glands, mononuclear cell infiltrations were observed in perivascular and periductal regions of all strains (Figures 1(a) to (c)). Notably, cell infiltrations in the pancreases of B6.MRLc1 and MRL/MpJ mice were more severe than that of the C57BL/6 strain (Figure 1(c)). In the pancreases of B6.MRLc1 and MRL/MpJ strains, cell infiltrations were also observed beside, but not inside of the pancreatic islets (Figure 1(c)). In our histometrical analysis of cell infiltration area, no significant strain-specific differences were detected in the salivary glands (Figure 1(d)). In the pancreas, the cell infiltration area was significantly larger in B6.MRLc1 and MRL/MpJ mice than in C57BL/6 animals (B6.MRLc1: $P = 0.013$, MRL/MpJ: $P = 0.009$, compared to C57BL/6) (Figure 1(d)). Because of these findings, subsequent analyses focused on the pancreatic pathology.

Histopathological characteristics of mononuclear cell infiltrates in mouse pancreases

In the pancreases of C57BL/6, B6.MRLc1, and MRL/MpJ strain mice at 9–12 months of age, mononuclear cell infiltrates were localized to the connective tissue between compressed pancreatic acini (Figure 2(a)). Although the borders between pancreatic acini and the cell infiltrations were defined in C57BL/6 mice, the borders were indistinct in B6.MRLc1 and MRL/MpJ strains because of the mononuclear cells migrating between the pancreatic acini (Figure 2(b), arrowheads).

In immunohistochemistry experiments, CD3- (T-cell marker) and B220- (B-cell marker) positive cells were observed in the cell infiltrations of all the strains examined. Notably, CD3- (Figure 2(c)) and B220-positive cells (Figure 2(d)) were localized to central and peripheral regions of infiltrates, respectively. There were no strain-specific differences in the localization of either marker. Further, LYVE1-positive lymph capillary was observed around/within the cell infiltrates of all the strains examined (Figure 2(e)).

Strain- and sex-specific differences in inflammatory cell infiltrates in mouse pancreases

The appearance of pancreatic cell infiltrates were semi-quantitatively scored in the pancreases of male and female mice belonging to nine strains including A/J, AKR/N, BALB/c, C3H/He, DBA/1, and DBA/2 at 5–7 months of age, and B6.MRLc1, C57BL/6, and

226 MRL/MpJ over the age of 6 months (Table 2). With the exception of BALB/c and C3H/He
227 mice, inflammatory cell infiltration was observed more frequently in females than in males.
228 Briefly, cell infiltration in perivascular or periductal regions (as shown in Figure 2(a)) was
229 more frequently observed in A/J, AKR/N, B6.MRLc1, C57BL/6, and MRL/MpJ strain
230 females and MRL/MpJ males. Further, a moderate amount of cell infiltration was observed
231 in DBA/1 and DBA/2 female mice, and AKR/N, B6.MRLc1, and C57BL/6 males. The cell
232 infiltration between the acini was observed only in females belonging to the A/J, AKR/N,
233 B6.MRLc1, DBA/1, and MRL/MpJ strains.
234
235 *Clinical parameters for pancreatitis, autoimmunity, and diabetes in mice*
236 The serum levels of the pancreatitis markers amylase and lipase, the autoimmunity
237 marker anti-dsDNA antibody, and blood glucose as a marker of diabetes were measured in
238 C57BL/6, B6.MRLc1, and MRL/MpJ female mice at 9–12 months of age (Table 3). The
239 serum amylase levels were significantly higher in B6.MRLc1 and MRL/MpJ strains than
240 in C57BL/6 mice (B6.MRLc1: P=0.025, MRL/MpJ: P=0.017, compared to C57BL/6). The
241 serum lipase level in B6.MRLc1 mice was higher than that of C57BL/6 and MRL/MpJ mice,
242 but this increase was not statistical significant. Similar to amylase levels, the serum level
243 of anti-dsDNA antibody was higher in B6.MRLc1 and MRL/MpJ strains than C57BL/6

mice (MRL/MpJ: P=0.001, compared to C57BL/6). The difference detected between MRL/MpJ and C57BL/6 strains was statistically significant. Anti-dsDNA antibody in B6.MRLc1 mice at 12–14 months (412.7 ± 108.3 μg/ml) was significantly higher than that of C57BL/6 mice at 9 months of age (P=0.009, compared to C57BL/6). The serum blood glucose level was not significantly different in the mouse strains we examined.

Investigation of acinar cell injury and fibrosis in mouse pancreases

It has been reported that pancreatic acinar cell injury induces the activation of the nuclear factor-kappa B pathway, resulting in the upregulation of downstream genes including interleukin 1 beta (*Il1b*), *Il6*, and tumor necrosis factor alpha (*Tnfa)* in mouse pancreatitis [19]. In the pancreas, the mRNA expression of the inflammatory cytokines *Il1b, Il6,* and *Tnfa* was compared in C57BL/6, B6.MRLc1, and MRL/MpJ female mice at 9－12 months of age (Figure 3(a)). The mRNA expression level of these genes tended to be higher in B6.MRLc1 and MRL/MpJ strain mice than in C57BL/6 mice. Notably, a statistically significant difference was detected in *Tnfa* expression (B6.MRLc1: P = 0.027, MRL/MpJ: P = 0.009, compared to C57BL/6) (Figure 3(a)).

To evaluate pancreatic acinar cell death and fibrosis in detail, TUNEL staining (Figure 3(b)) and immunohistochemistry for αSMA (Figure 3(c)) were performed in C57BL/6,

B6.MRLc1, and MRL/MpJ females at 9−12 months of age. Many TUNEL-positive nuclei were detected in the acinar cells near and in the cell infiltrations in B6.MRLc1 and MRL/MpJ mice (Figure 3(b), arrows and arrowheads, respectively). In contrast, TUNEL staining was only detected in the cell infiltrates in C57BL/6 mice (Figure 3(b), arrowhead). Immunohistochemistry for αSMA demonstrated that αSMA-positive cells were detected only in vascular and ductal walls (Figure 3(c)).

Pancreatic expression of immune-associated genes present on the telomeric region of mouse chromosome 1

We investigated the mRNA expression of immune-associated genes on *Mag*, including *Sel* and *Fcgr* family members (Figure 4(a)), in C57BL/6, B6.MRLc1, and MRL/MpJ female mice at 9–12 months of age. No significant strain-specific expression differences were detected in the three *Sel* genes examined (Figure 4(b)). Further, the high endothelial venule (HEV) marker PNAd, a sugar chain and binding partner of L-selection (encoded by *Sell*), was observed only in cell infiltration areas. However, no significant difference was observed in the localization and number of PNAd-positive cells among the three strains (Figure 4(c)). In addition, pancreatic *Fcgr2b* expression was not significantly different in the three strains (Figure 4(d)). The expression level of *Fcgr3*, as well as the ratio of *Fcgr3* to *Fcgr2b*

280 expression, an indicator of immune response activation, was markedly and significantly
281 higher in B6.MRLc1 and MRL/MpJ strains than in C57BL/6 mice that have an undetectable
282 level of *Fcgr3* expression (*Fcgr3*: B6.MRLc1: $P = 0.001$, MRL/MpJ: $P = 0.003$; *Fcgr3/Fcgr2b*:
283 B6.MRLc1: $P = 0.005$, MRL/MpJ : $P = 0.002$, compared to C57BL/6) (Figure 4(d)).
284

Discussion

Pathology of chronic pancreatitis mediated by the MRL/MpJ genetic background

In the salivary glands, cell infiltrates were detected in all mice examined, but there was no difference among strains. In contrast, cell infiltration in the pancreases was significantly more prevalent in B6.MRLc1 and MRL/MpJ than C57BL/6 strain mice. Based on the serum autoantibody and amylase levels, and the mRNA expression of inflammatory- and autoimmune-associated genes in pancreases of these mouse strains, we concluded that B6.MRLc1 and MRL/MpJ mice develop autoimmune-mediated pancreatitis. The mouse genetic background affects the autoimmune phenotype, a difference that has also been reported for the Th response. Mouse strains including C57BL/6 and DBA/1 favor the Th1 response, while other strains, such as DBA/2, BALB/c, AKR/N, and MRL/MpJ, favor the Th2 response.[20-23] In this study, there was no consistent relationship between Th biases and cell infiltrations in the mouse pancreas.

Pancreatic cell infiltrations were frequently detected in B6.MRLc1 and MRL/MpJ as well as C57BL/6, A/J, and AKR/N strains. Interestingly, C57BL/6 and AKR/N are ancestor strains of MRL/MpJ.[10] Although there is no etiological report regarding cell infiltration in the pancreases of C57BL/6, A/J, and AKR/N mice, a recent study reported that C57BL/6 developed age-related exocrinopathy in the salivary glands at approximately 90 weeks of

303 age.[24] Females tended to show pancreatic cell infiltration more frequently than males in
304 this study. It has been suggested that estrogen and progesterone exacerbate the
305 autoimmune response in both humans and mice,[25] and testosterone acts as a protective
306 factor in the autoimmune phenotype of B6.MRLc1 mice.[14] Taken together, *Mag*, the
307 C57BL/6 background, and the action of sex hormones affected the development of
308 autoimmune-mediated pancreatitis in this study.
309
310 *Pathological type of pancreatitis in B6.MRLc1 and MRL/MpJ*
311 In SS, the inflammatory lesion is predominantly localized to the salivary glands.[26] In
312 AIP, Type1 shows an infiltration of lymphocytes and plasma cells and elevated serum
313 autoantibodies, while Type2 is associated with the infiltration of granulocytes without the
314 presence of serum IgG4.[6] The pathological features of the pancreatitis in B6.MRLc1 and
315 MRL/MpJ were consistent with that of Type1 AIP rather than SS or Type2 AIP. However,
316 there were some pathological differences between typical Type 1 AIP patients and the
317 model mice used in this study. Type1 AIP is predominantly observed in male patients and
318 patients possess prominent interlobular fibrosis,[27] but these characteristics of AIP were
319 not apparent in B6.MRLc1 and MRL/MpJ strain mice. Furthermore, Type1 AIP is an
320 IgG4-related human disease, while mice lack this IgG-subtype.[21] Similar to patients with

SS and AIP, patients with systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) also exhibit inflammation of the exocrine glands to some extent.[28] These autoimmune diseases are polygenic disorders, for which several genetic loci are involved in the development of symptoms. At least 53 susceptibility loci are reported in SS.[29] In fact, *Mag*-derived pancreatitis was mild and lacked the fibrosis that is typically observed in AIP and AIP susceptibility loci are also reported on chromosomes 2 and 6 in other mouse models.[12] Therefore, *Mag* might be a MRL/MpJ-derived susceptibility locus for autoimmune-mediated pancreatitis, but other susceptibility loci might be needed to completely mimic the representative phenotype of autoimmune diseases including SS, AIP, and SLE.

Pathogenesis of pancreatitis in B6.MRLc1 and MRL/MpJ

Mag encoded several immune-associated genes[13], especially Ifi200 family is a candidate of the development of autoimmune disease in mice.[30] Whereas, our previous study showed that the mRNA expression levels of Ifi200 family (*Ifi202*, *Ifi203*, *Ifi204*, *Ifi205*) in the pancreases were low in both C57BL/6 and B6.MRLc1.[31] In this study, we focused and examined the expression of *Sel* and *Fcgrs* genes encoded on *Mag* as candidate factors that contribute to pancreatitis in B6.MRLc1 and MRL/MpJ. Although the cell

infiltrates participated in the formation of periductal lesions through the interaction of HEVs expressing PNAd and leukocytes expressing L-selectin,[32] we did not identify a pathological relationship between cell infiltration and localization, the number of PNAd-positive HEVs, and the mRNA expression levels of *Sel* genes in the pancreas of B6.MRLc1 mice. However, LYVE1-positive lymph capillaries were also observed around the cell infiltration in pancreases, and we considered that HEV and lymph capillaries might act as the gates for cell infiltrations.

Importantly, the mRNA expression of *Fcgr3* and the *Fcgr3 to Fcgr2b* expression ratio were drastically elevated in the pancreases of B6.MRLc1 and MRL/MpJ mice. FcγRs are expressed in various immune cells, and altered functions and affinities with immune-complex in inhibitory type FcγRIIB were found in autoimmune diseases such as SLE in both humans and mice.[14] Previous studies also suggested that high expression of *Fcgr3,* low expression of *Fcgr2b*, and the altered of balance of the *Fcgr3/Fcgr2b* expression ratio were associated with the development of autoimmune glomerulonephritis, splenomegaly, and high serum anti-dsDNA antibody.[13] Recently, AIP patients with autoimmune glomerulonephritis were reported.[33,34] In addition, polymorphisms in *Fcgr* genes have been reported in human patients with autoimmune diseases including SS, SLE, RA, and multiple sclerosis.[35] Therefore, of the genes

encoded in *Mag*, the altered expression of *Fcgrs* might be crucial as the cause of autoimmune-mediated pancreatitis.

Important pathological features including the invasion of cells between acini, TUNEL-positive acinar cells near cell infiltrations, and elevated cytokine mRNA levels, particularly *Tnfa*, were observed in B6.MRLc1 and MRL/MpJ mice, but not in the C57BL/6 strain. It has been reported that Tnfα protein, produced by the binding of substance P and neurokinin 1 receptor in acinar cells, induces acinar cell death and exacerbates cell infiltration through the upregulation of intercellular adhesion molecule 1 in vascular endothelial cells.[36] It is also reported that FcγRIII and immune-complex induced Tnfα production in alveolitis model mice,[37] and immune-complex depositions were observed in the glomerulus of B6.MRLc1.[13] Therefore, we hypothesize that *Fcgr3* upregulation and its activation by immune-complexes might contribute to acinar cell injury through the production of inflammatory mediators including Tnfα.

In conclusion, B6.MRLc1 might be useful for the analysis of the molecular pathogenesis of autoimmune-mediated pancreatitis and this study emphasized the pathological importance of *Mag* locus in this disease.

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379

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487 **Table 1. Summary of the specific gene primers in this study**

Gene(accession no.)		Primer sequence	Product size (bp)
Actb (NM_007393)	Forward	ACTGCTCTGGCTCCTAGCAC	196
	Reverse	CAGCTCAGTAACAGTCCGCC	
Sele (NM_011345)	Forward	ACTTCAGTGTGGTCCAAGAGG	156
	Reverse	CATTGAAAGGCACATGAGGAC	
Sell (NM_011346)	Forward	TCTTCATTCCTGTAGCCGTCA	246
	Reverse	CGTTGAGCAAACACTGCATC	
Selp (NM_011347)	Forward	CAGGGACACTGACAATCCAG	234
	Reverse	CGAATGAGACATTGGCAGAC	
Il1b (NM_008361)	Forward	AAGGAGAACCAAGCAACGAC	208
	Reverse	AACTCTGCAGACTCAAACTCCAC	
Il6 (NM_031168)	Forward	TGTATGAACAACGATGATGCAC	137
	Reverse	TGGTACTCCAGAAGACCAGAGG	
Tnfa (NM_013693)	Forward	CGAGTGACAAGCCTGTAGCC	167
	Reverse	GAGAACCTGGGAGTAGACAAGG	
Fcgr2b (NM_010187)	Forward	TGCAGCTGACTCGCTCCAGA	123
	Reverse	CACGGCTGTCCACAGTAGCA	
Fcgr3 (NM_010188)	Forward	ATCACTGTCCAAGATCCAGC	353
	Reverse	GCCTTGAACTGGTGATCCTA	

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490 **Table 2. Strain difference of pancreatic inflammatory cell infiltration**

	Cell infiltrations in perivascular or periductal regions		Cell infiltrations between acini	
	Female	Male	Female	Male
MRL/MpJ	＋＋	＋＋	＋	－
B6.MRLc1	＋＋	＋	＋	－
C57BL/6	＋＋	＋	＋	－
AKR/N	＋＋	＋	＋	－
A/J	＋＋	－	＋	－
DBA/1	＋	－	＋	－
DBA/2	＋	－	－	－
BALB/c	－	－	－	－
C3H/He	－	－	－	－

Pancreas from 4 mice from each strain are investigated and scored from – to ++.
－ : no infiltration cell is observed in all mice. + : infiltration cells are observed in 1 or 2 mice. ++ : infiltration cells are observed in 3 or 4 mice.

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492

493 **Table 3. Clinical parameters for pancreatitis, autoimmunity, and diabetes in mice**

Parameters	C57BL/6	B6.MRLc1	MRL/MpJ
Amylase (U/ml)	3360±101	26514±17387 *	4048±208 *
Lipase (U/ml)	210±37	548±215	227±33
Anti-dsDNA antibody (μg/ml)	101±17	187±60	357±20 *
Blood glucose (mg/dl)	249±36	191±26	257±69

n ≥ 3. Values = mean ± SE. *: significant difference compared to
494 C57BL/6 ($P < 0.05$, non-parametric Mann-Whitney U test)

495

Figure legends

Figure 1. Histopathologies of mouse exocrine glands.

(**a**)–(**c**) The parotid glands (a), sublingual glands (b), and pancreases (c) of C57BL/6, B6.MRLc1, and MRL/MpJ female mice examined at 9–12 months of age. The area surrounded by the dotted line shows mononuclear cell infiltration. Cell infiltrations are primarily observed at the perivascular and periductal regions of the exocrine glands. The areas of cell infiltration are the largest in the pancreases of B6.MRLc1 and MRL/MpJ mice (c). Sections are stained with HE. Bars = 100 μm. (**d**) The area of cell infiltration in parotid glands, sublingual glands, and pancreases of C57BL/6, B6.MRLc1, and MRL/MpJ female mice examined at 9－12 months of age. $n \geq 5$. Values = mean ± SE. *: significant difference from C57BL/6 ($P < 0.05$).

Figure 2. Characteristics of cell infiltrates in mouse pancreases.

(**a**) Cell infiltrations in the pancreases of C57BL/6, B6.MRLc1, and MRL/MpJ female mice examined at 9－12 months of age. Mononuclear cell infiltrations are observed in the connective tissue between the pancreatic acini and localized near pancreatic islets. (**b**) Enlarged images of the square region in (a). The arrowheads indicate the border between infiltrations and normal pancreatic acini. These borders are distinct in C57BL/6 mice. In

514 B6.MRLc1 and MRL/MpJ, mononuclear cells invade between the pancreatic acini, and the
515 borders between infiltrations and normal pancreatic acini are indistinct. (**c**) The localization
516 of CD3-positive T-cells in cell infiltrations. (**d**) The localization of B220-positive B-cells in cell
517 infiltrations. (**e**) The localization of LYVE1-positive lymph capillaries. C57BL/6, B6.MRLc1,
518 and MRL/MpJ female mice were examined at 9－12 months of age. The pancreas sections
519 were analyzed by immunohistochemistry. The CD3- and B220-positive cells localize to center
520 and marginal regions of the cell infiltrates, respectively. LYVE1-positive lymph capillaries
521 are observed around/within the cell infiltrations (arrows). Bars = 100 μm.
522
523 **Figure 3.** Evaluation of pancreatic acinar cell injury and fibrosis in mouse pancreases.
524 (**a**) The pancreatic mRNA expression level of mouse *Il1b*, *Il6*, and *Tnfa*. C57BL/6, B6.MRLc1,
525 and MRL/MpJ female mice were examined at 9–12 months of age. $n \geq 3$. Values = mean ± SE.
526 *: significant difference from C57BL/6 ($P < 0.05$). (**b**) The localization of TUNEL-positive
527 cells. C57BL/6, B6.MRLc1, and MRL/MpJ female mice were examined at 9–12 months of age.
528 TUNEL-positive cells are detected in the pancreatic acinar cells near cell infiltrations
529 (arrows) and in the infiltrating cells themselves (arrowheads) in B6.MRLc1 and MRL/MpJ
530 mice. TUNEL-positive cells were found only in the infiltrating cells in C57BL/6 mice. (**c**) The
531 localization of αSMA-positive cells. C57BL/6, B6.MRLc1, and MRL/MpJ female mice were

examined at 9 − 12 months of age. The pancreas sections were analyzed by immunohistochemistry. The αSMA-positive cells are detected in the vascular and ductal wall in the mice examined. Bars = 100 μm.

Il: interleukin; *Tnfa*: tumor necrosis factor alpha; TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling; αSMA: alpha smooth muscle actin.

Figure 4. Pancreatic expression of mouse selectin and Fc gamma receptor family genes. (**a**) A schematic of the congenic region on B6.MRLc1 chromosome 1. Black and white boxes indicate the chromosome regions derived from MRL/MpJ and C57BL/6 strains, respectively. (**b**) The pancreatic mRNA expression level of *Sele*, *Sell,* and *Selp.* C57BL/6, B6.MRLc1, and MRL/MpJ female mice were examined at 9–12 months of age. n ≥ 3. Values = mean ± SE. n.d.: not detected. (**c**) The localization of PNAd in mouse pancreases. C57BL/6, B6.MRLc1, and MRL/MpJ female mice were examined at 9–12 months of age. The pancreas sections were analyzed by immunohistochemistry. The PNAd-positive cells (arrowheads) are detected in cell infiltrations (dotted lines) in all the mice examined. Bars = 100 μm. (**d**) The mRNA expression level of *Fcgr2b* and *Fcgr3* and the expression ratio of *Fcgr3*/*Fcgr2b*. C57BL/6, B6.MRLc1, and MRL/MpJ female mice were examined at 9–12 months of age. n ≥ 3. Values = mean ± SE. n.d.: not detected. *: significant difference from C57BL/6 ($P < 0.05$).

550 *Sele*: selectin endothelial cell; *Sell*: selectin lymphocyte; *Slep*: selectin platelet (*Selp*); *Fcgr2b*:

551 Fc receptor IgG low affinity IIb; *Fcgr3*: Fc receptor IgG low affinity III; PNAd: peripheral

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