MOLECULAR, IMMUNOLOGICAL AND GENETIC STUDIES ON MURINE AUTOIMMUNE PANCREATITIS

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Vorgelegt von:

Farahnaz Asghari, M.Sc.
geboren am 08.12.1967 in Hashtrood (Iran)

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Gutachter:

1.Gutachter:

Prof. Dr. med. Robert Jaster
Klinik für Innere Medizin II
Abteilung Gastroenterologie und Endokrinologie,
Universitätsmedizin Rostock

2.Gutachter:

Prof. Dr. Ulrike Gimsa
Leibniz-Institut für Nutztierbiologie
Institut für Verhaltensphysiologie, Dummerstorf

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1 Introduction

1.1 Autoimmunity

The key role of the immune system is to distinguish self-antigens (autologous) from foreign agents such as bacteria, viruses or various environmental factors that are recognised as non-self (pathogens). Immune system reacts specifically against pathogens but acquires self-tolerance to autologous antigens. Tolerance to self-antigens is acquired through several distinct regulatory mechanisms such as clonal deletion of auto-reactive T clones in the thymus during the prenatal period or functional suppression of auto-reactive T and B clones at later stages of the development of the organism (anergy, ignorance and suppression). A failure to distinguish 'self' from 'non-self' may lead to an autoimmune response, which is characterised by activation and clonal expansion of autoreactive T and B-lymphocytes, and production of autoantibodies. This in turn results in inflammation, tissue injury and subsequent development of autoimmune diseases, such diabetes mellitus type1, Graves' disease, Hashimoto's thyroiditis, Idiopathic Thrombocytopenic Purpura (ITP), Rheumatoid Arthritis (RA), Sjögren's syndrome, Systemic Lupus Erythematosus (SLE) and autoimmune pancreatitis (AIP).

1.2 Autoimmune Diseases (AID)

Autoimmune diseases can be classified into two distinct groups: organ specific (localised) and non-organ specific (systemic autoimmune disorders). The effects of systemic autoimmune diseases present throughout the entire body, i.e. commonly showing inflammation in joints, muscles and organs. The prevalence of autoimmune disorders is particularly reported in Europe and the USA. The National Institute of Health (NIH) estimated approximately 14.7-23.5 million Americans to be affected with at least one autoimmune condition (Autoimmune Diseases Coordinating, 2005). According to the American Autoimmune Related Diseases Association (AARDA), it has been estimated that approximately 50 million people are affected with AID in the United States (Schmidt, 2011), of which 75% are women. Based on these reports, the incidence of autoimmune diseases is 8% in the USA population (Autoimmune Diseases Coordinating, 2005) and from 3-5% worldwide (Jacobson et al., 1997; Maas et al., 2002; Marrack et al., 2001) therefore, further indicating that the prevalence of AID had extremely increased over the past few years.

The susceptibility to complex diseases, such as autoimmune and chronic inflammatory disorders is known to be controlled by genes, environmental factors, or interaction between both (Bang et al., 2010; Franke et al., 2010; Han et al., 2009; Naldi et al., 2005). Over the last decade the genetic basis of many autoimmune diseases have been extensively studied (Fernando et al., 2008).

1.2.1 Autoimmune Pancreatitis (AIP)

1.2.1.1 General Aspect of Autoimmune Pancreatitis

Chronic pancreatitis (CP) is a relapsing inflammatory disease that leads to permanent degeneration, exocrine-endocrine dysfunction of the pancreas, and an increased risk of pancreatic cancer (Etemad & Whitcomb, 2001). Auto-digestive reactions including secretion of pancreatic proteases and subsequent regenerative processes, play a pivotal role in the pathogenesis of the disease (Klöppel & Maillet, 1993). The major risk factor for developing CP includes alcohol abuse. However, biliary diseases and genetic defects are also known to be a common cause of CP. Interestingly, in up to one third of the cases, the cause of CP remains unknown. Chronic pancreatitis usually develops in adults aged 40 to 50 and it predominantly affects more men than women (Etemad & Whitcomb, 2001). It has been previously reported that alcoholic pancreatitis is the most common type of CP in males, while idiopathic pancreatitis is reported to affect predominantly females (Lin et al., 2000). Despite on the growing knowledge in field, the treatment of CP remains challenging in vast majority of the cases.

AIP is a rare cause of chronic pancreatitis that was recognized as a clinical entity in the last decades. The first description of chronic pancreatitis with sclerosis as an autoimmune condition was published in 1961 (Sarles et al., 1961). Thirty years later Kawaguchi reported two cases of unusual inflammatory disease involving pancreas and biliary tract and described it as a lymphoplasmacytic sclerosing pancreatitis (LPSP) (Kawaguchi et al., 1991). However, the original term of “autoimmune pancreatitis” was proposed only later by Yoshida et al. (Yoshida et al., 1995). According to a study performed in a regional cohort of 545 CP patients with, the percentage of AIP in all forms of CP is approximately 10% (Schneider & Löhr, 2009).

Occasionally, AIP is associated with other autoimmune diseases, primarily the Sjögren's syndrome (Kamisawa, Funata, Hayashi, Tsuruta et al., 2003; Pickartz et al., 2004). Histopathologically, AIP is characterised by a periductal inflammatory cell infiltration, followed by an obliterative periductal fibrosis (Klimstra & Adsay, 2004). In severe cases, the inflammation includes pancreatic acini, which lead to the loss of lobular pancreatic architecture. Both inflammation and fibrosis may result in the formation of an inflammatory pseudotumour (Klöppel et al., 2003) and cause obstructive jaundice, thus making the differential diagnosis between AIP and pancreatic carcinoma difficult. As a result, up to 10% of patients who undergo surgical resection for a suspected pancreatic cancer have a final diagnosis of pancreatitis (Wolfson et al., 2005). Usually AIP patients respond well to corticosteroid therapy, whereas the application of steroids is ineffective in more common forms of CP (Kojima et al., 2003; Song et al., 2005).

One of the key features of AIP are elevated IgG4 levels in AIP patients sera (Wang et al., 2013). Over the last years, the variable sensitivity of IgG4 levels in the sera of the patients (from 70% to 90.9%) was proposed as a diagnostic marker for classification of AIP into its distinct subtypes, AIP 1 and 2. Moreover, high concentrations of IgG4 in patients serum provided a useful tool for differential diagnosis of sclerosing pancreatitis from other diseases of the pancreas or biliary tract (Hamano et al., 2001). Despite the identification of autoantibodies in AIP, IgG4 sera content is still not considered as a specific diagnostic marker (Frulloni et al., 2009). Hence, more sensitive and specific markers for diagnosis of AIP are required.

To date, the first diagnostic criteria for AIP were issued by the Japan pancreas society (JPS) in 2002. During last decade, at least eight sets of diagnostic criteria of AIP have been proposed (Otsuki et al., 2008). However, the criteria summarized as "HISORt" and "The Final Asian Criteria" are currently the main criteria for the diagnosis of AIP (Chari, 2007; Divatia et al., 2012).

Mayo clinic diagnostic criteria for autoimmune pancreatitis summarised as HISORt (Chari, 2007) and are based on the following five cardinal features:

- Histology: at least one of the following features: Lymphoplasmacytic infiltrate with storiform fibrosis and obliterative phlebitis (LPSP) or LPSP abundant IgG4 cells (≥ 10 IgG4 cells/ HPF)[1]

[1] HPF; per high power field

- Imaging: "sausage-shaped" enlargement of the gland on a computerized tomography (CT) scan and magnetic resonance imaging (MRI)
- Serology: elevated titers of gammaglobulins (IgG)[2], elevated serum levels of the IgG4 and a variety of antibodies including antinuclear antibody, rheumatoid factor, carbonic anhydrase, and lactoferrin
- Other organ involvement: biliary ductal system, salivary glands and retroperitoneum
- Response to steroid therapy

Considering these criteria, AIP should be suspected in patients with obstructive jaundice, pancreatic mass/enlargement or pancreatitis, and patients who exhibit one or more of these five cardinal features.

The Final Asian AIP diagnostic criteria were obtained at the third Japan–Korea symposium on AIP in 2008, based on following features:

I: Imaging (both required): **a:** enlargement of the gland (diffuse/ segmental/ focal) occasionally with a mass and/or a hypo-attenuation rim, **b:** pancreatic duct narrowing (diffuse/ segmental/ focal) often with stenosis of the bile duct.

II: Serology (one required): elevated serum IgG/ IgG4 and detectable autoantibodies (ANA; Anti-nuclear antibody and RF; Rheumatoid factor).

III: Histopathology: lymphoplasmacytic infiltration (IgG4 positive) with fibrosis.

It has been proposed that AIP should be diagnosed when criterion I (Ia and Ib) and one of the other two criteria II or III are fulfilled (Criterion I+II, Criterion I+III, Criterion I+II+III) or when lymphoplasmacytic sclerosing pancreatitis in the resected pancreas is observed (Otsuki et al., 2008).

International consensus diagnostic criteria (ICDC) were developed for diagnosis of both subtypes of AIP separately. The combination of one or more of following five cardinal features; namely, imaging of pancreatic parenchyma and duct, serology, other organ involvement, pancreatic histology and an optional criterion of response to steroid therapy could be used to confirm AIP diagnosis. Each feature was classified into level 1 and 2 based on the diagnostic reliability (Otsuki et al., 2008; Park, Chung et al., 2008).

[2] Ig stands for immunoglobulin

Recently, the diagnostic criteria for AIP were expanded due to the accumulation of clinical experience, development of newer diagnostic methods and changes in the concept of AIP. The differentiation of AIP from pancreatic cancer is extremely important. In this regard, there is a need to update the criteria continuously and to develop unified criteria, which would be shared worldwide. In accordance with this purpose, Ebbo et al. reviewed the pathophysiological hypothesis and therapeutic approaches of IgG4-RSD and suggested that there is still no international validated diagnostic criteria for IgG4-RSD (Ebbo et al., 2012).

Most of autoimmune pancreatitis cases have been reported from Japan, Europe and the US. The disease descriptions from Japan have been based mainly on the existence of different clinical phenotypes. On the other hand, reports from Europe and the USA described at least two distinct histopathological patterns found in AIP patients; lymphoplasmacytic sclerosing pancreatitis (LPSP; type 1); and idiopathic duct-centric pancreatitis (IDCP; type 2) or granulocytic epithelial lesion (GEL)-positive pancreatitis. The two subtypes can be distinguished by their development of extrapancreatic manifestations, namely periductal lymphoplasmacytic infiltration and peculiar periductal fibrosis(Chari et al., 2010; Okazaki et al., 2010). The characteristic findings of type AIP type1 are sufficient infiltration of pancreatic tissue by $IgG4^+$ plasma cells and lymphocytes (Masaki et al., 2011), storiform fibrosis, association with non-specific autoantibodies and elevated serum IgG4/IgE levels. In addition, these patients often show extrapancreatic lesions such as sclerosing cholangitis, sclerosing sialadenitis, or retroperitoneal fibrosis with similar pathological features. Based on these findings, some synonyms were suggested for the same condition, namely “multifocal idiopathic fibrosclerosis”, “IgG4-related autoimmune disease”, “IgG4-related sclerosing disease”, “IgG4-related plasmacytic disease”, and “IgG4-related multiorgan lymphoproliferative syndrome” (Kamisawa, Funata, Hayashi, Tsuruta et al., 2003; Ketwaroo & Sheth, 2013; Okazaki et al., 2011; L. Zhang & Smyrk, 2010). As opposed to AIP type 1, AIP type 2 is predominantly characterised by granulocytic epithelial lesions, which are frequently associated with a destruction and obliteration of the pancreatic duct. Moreover, no elevated IgG4 levels and infiltrates of IgG4-positive plasma cells were observed in the sera of patients suffering from AIP type 2 (Table 1.1). Finally, an association between inflammatory bowel disease and AIP type 2 has been also previously described (Okazaki et al., 2010). Up to date, the study of pathogenesis of the different subtypes of AIP in mice remains difficult due to the lack of IgG4 in mice. Nevertheless, the presence of nonspecific autoantibodies in the serum and the histology of the pancreas, specifically the absence of granulocyte epithelial

lesions in the pancreatic ducts and acini are just some of the evidence that show that MRL/Mp mouse is a good model to study the human AIP type 1.

Table 1.1 Comparison of type1 and type2 autoimmune pancreatitis (AIP)

	Type 1 AIP	Type 2 AIP
Synonym	Lymphoplasmacytic sclerosing pancreatitis AIP without GEL	Idiopathic duct-centric chronic pancreatitis AIP with GEL
Epidemiology	Asia> USA, Europe	Europe> USA> Asia
Age at diagnosis	Old	Young (Child and adult)
Gender predominantly	Male predominance	Almost equal or small difference
Clinical presentation	Obstructive jaundice (painless)	Obstructive jaundice (painless), abdominal pain/acute pancreatitis
Serum IgG4 levels	Often Elevated	Normal
Other organ involvement	Gland, kidney, retoperitoneum	Not seen
Histological nomenclature	Lymphoplasmacytic sclerosing pancreatitis	Idiopathic duct-centric Pancreatitis
Ducts	Periductal inflammation Preserved epithelium	Intraepithelial neutrophils damaged epithelium
Lobule	Lymphoplasmacytic inflammation with storiform fibrosis	Patchy inflammation, often with neutrophils
Veins	Phlebitis very common	Phlebitis unusual
$IgG4^+$ plasma cells	Many	Rare
Extra-pancreatic lesions	IgG4-RD	Inflammatory bowel disease
Granulocytic epithelial lesion	Absent	Present
Association with ulcerative colitis	Occasionally	Common
Steroid responsiveness	Excellent	Excellent
Relapse rate	High	Low

AIP, autoimmune pancreatitis; GEL, granulocytic epithelial lesion; IgG4-RD, IgG4-related disease. Adopted from (Kamisawa et al., 2013; Okazaki et al., 2011; Park et al., 2009; Wang et al., 2013).

1.2.1.2 Epidemiology

The prevalence of autoimmune pancreatitis (AIP) is reported regularly from Japan, Europe and the USA (Chari et al., 2010). Although recent worldwide reports show that the incidence and the prevalence of autoimmune diseases are rising (Wang et al., 2013), the prevalence of AIP in the world remains unknown. Recent data showed that the prevalence and the incidence of AIP in Japan is 2.2 and 0.9 cases per 100,000 population, respectively (Kanno et al., 2012). AIP type I predominantly affects male patients with a male/female ratio of 2-5:1 in Japan and 2:1 in Europe (Okazaki et al., 2005) whereas type 2 AIP shows no male predominance (Kamisawa et al., 2013). The age at diagnosis is 60-70 years in patients with type 1 AIP, and 50-60 in patients with type 2 AIP (Chari et al., 2010; Detlefsen et al., 2011; Nishimori et al., 2007; Okazaki et al., 2010).

1.2.1.3 Diagnosis

1.2.1.3.1 Clinical Manifestations

AIP is identified incidentally, because patients show no symptoms or only have mild symptoms and usually without acute attacks of pancreatitis. The most common clinical manifestation in AIP patients is obstructive jaundice with little or no abdominal pain, whereas abdominal pain and elevated serum pancreatic enzymes are frequently observed in type 2 AIP (Kamisawa, Chari et al., 2011). The main symptom of other forms of CP is severe acute pain attacks, which is rather rare in AIP. A severe abdominal pain, pancreatic calcification and cysts are also rare in AIP patients. The common characteristic findings in AIP are elevated serum gammaglobulin IgG/ IgG4, presence of autoantibodies, diffuse enlargement of the pancreas, diffusely irregular narrowing of the main pancreatic duct, fibrotic changes with lymphocyte infiltration, occasional association with other autoimmune diseases and effective steroid therapy (Okazaki & Chiba, 2002). AIP, as a multi-organ disorder, presents both pancreatic and extrapancreatic features. Several extrapancreatic lesions that were reported in AIP patients are sclerosing cholangitis, sclerosing sialadenitis and retroperitoneal fibrosis. Pancreatic exocrine or endocrine malfunction in AIP, leads to diabetes mellitus (DM) or diabetes and steatorrhoea (Kamisawa et al., 2013; Okazaki, 2005). It has been reported that patients with AIP had DM prior to AIP or develop it simultaneously with AIP (Kamisawa, Egawa et al., 2003). AIP patients may show some symptoms that are associated to other

diseases, such as impaired glucose tolerance in patients with DM (Wang et al., 2013). The manifestations of AIP are versatile in both subtypes of AIP and are summarised in Table 1.1.

1.2.1.3.2 Pathology

Different pathological features of type 1 and type 2 AIP are more important for the diagnosis than an elevated serum IgG4 concentration. Pathological findings in type 1 AIP are IgG4-positive plasma cells and T-lymphocytes infiltration, storiform fibrosis, obliterative phlebitis and eosinophil infiltration, whereas neutrophilic infiltration in or around the pancreatic duct, duct destruction and rare obliterative phlebitis are common in type 2 AIP (Kamisawa et al., 2012; Wang et al., 2013).

1.2.1.3.3 Laboratory Findings

The characteristic laboratory findings in patients with AIP are: elevated serum gammaglobulin IgG/ IgG4, presence of auto-antibodies, increased hepatobiliray or pancreatic enzymes and impaired exocrine, and endocrine function of pancreas (Okazaki et al., 2005). Elevated serum pancreatic enzymes, namely lipase and amylase are detected in AIP type 2 patients (Aughsteen et al., 2005; Tran et al., 2012). Although elevated serum IgG4 concentration has been reported in most patients with AIP type 1, it is not a specific diagnostic marker for AIP (Strehl et al., 2011).

Several AIP-related autoantibodies have been reported in AIP patients, such as anti-lactoferrin (Okazaki et al., 2000), anti-carbonic anhydrase-II/IV (Aparisi et al., 2005; Nishimori et al., 2005), anti-pancreatic secretory trypsin inhibitor (Asada et al., 2006), anti-amylasealpha (Endo et al., 2009), autoantibodies against the plasminogen-binding protein (Frulloni et al., 2009). However, the clinical significance of the aforementioned antibodies in AIP remains elusive and there is still a need for more sensitive and specific markers to diagnose the disease.

1.2.1.4 Treatment

Steroid therapy (as a standard therapy) is clinically, morphologically and serologically effective for AIP patients regardless of their subtypes (Kamisawa et al., 2012; Kamisawa, Takuma et al., 2011). The main goal of steroid therapy in AIP is to induce remission. Various regimens of steroids are available, but their dosage and duration in the acute phase of AIP are

still debated. Kamisawa et al. reported that AIP patients who received steroid treatment have shown significantly higher remission and lower relapse rates than the control group (Kamisawa et al., 2009). Both subtypes of AIP respond well to glucocorticoid therapy (Shinagare et al., 2012), but relapse rate is significantly higher in type 1 AIP than in type 2 AIP patients (Detlefsen et al., 2011; Notohara et al., 2003). Predominantly, the response to therapy is defined by resolution of symptoms and radiological abnormalities as well as improvement in clinical and biomedical parameters (Ketwaroo & Sheth, 2013). Interestingly, splenomegaly in AIP patients was minimized after steroid therapy (Matsubayashi et al., 2013). Ordinarily, pancreatic function and morphologic characteristics return to normal within 4-6 weeks, whereas serological and radiological remissions require more time (Vlachou et al., 2011).

AIP patients who relapse during maintenance steroid therapy or after stopping steroid medication should be re-treated with a high-dose steroid (Kamisawa & Okamoto, 2007). Immunosuppressive therapy and biological agents are the alternative treatments for the patients with recurrent or refractory AIP. Immunosuppressive components such as Azathioprine, 6-Mercaptopurine (Ghazale et al., 2008), Mycophenolate mofetil (Boehm & Bieber, 2001; Pisoni et al., 2005) and Cyclophosphamide (Brodsky, 2002) have been examined besides or instead of steroid therapy. However, data about their efficacy and treatment course are limited (Ketwaroo & Sheth, 2013). Similar to AIP patients, MRL/Mp mice respond well to steroid therapy. In addition to steroids, alternative immunosuppressant agents such as “Rapamycin” and “Cyclosporine A” are effective in treatment of murine AIP (Schwaiger et al., 2013). Considering the key role of autoreactive T cells in the pathogenesis of AIP in the MRL/Mp mouse model, “Cyclosporine A” and “Rapamycin” were suggested as a new treatment of AIP in humans (Schwaiger et al., 2013). Using biological agents, namely B-cell depletion with Rituximab, could be a promising treatment in patients with IgG4- related systemic disease (IgG4-RSD), who have shown recurrent or prove refractory to conventional immunosuppressive therapy (Khosroshahi et al., 2010). Even Bortezomib, a proteasome inhibitor, is a successful therapy for IgG4-RD (Khosroshahi & Stone, 2011) but it’s efficacy for treating AIP is unforeseeable (Wang et al., 2013).

As the treatment options of AIP are currently limited and disease relapse is frequent, Seleznik et al proposed neutralizing LT-βR ligands, as a new therapeutic strategy based on data generated from their newly established mouse model for AIP. In their work, they have demonstrated that an overexpression of lymphotoxin (*LT*) α and β in the acinar cells

(Ela1-LTab mice) may lead to the development of an autoimmune condition strongly resembling the human AIP (Seleznik et al., 2012).

1.2.1.5 Environmental Risk Factors

Several important environmental risk factors such as allergy, radiation, viral or bacterial infections, alcohol and smoking, play an important role in the development of autoimmune diseases (Ai et al., 2003; Prummel et al., 2004). The interaction between smoking and some genes provide a high risk for development of autoimmune diseases (Bang et al., 2010; Plenge et al., 2007). Although smoking and alcohol consumption are known to be risk factors in CP, their contribution to the development of AIP is still unknown (Andriulli et al., 2010; Coté et al., 2011; DiMagno & DiMagno, 2003; Law et al., 2010; Yadav et al., 2009).

The association between particular vaccination and autoimmune diseases has been reported in few rare cases. For example, in Guillain-Barré syndrome (GBS), it was suggested that sensitization by microbes may lead to autoimmune response to the targeted system of the host, which is based on the homology of amino acid between the gangliosides of the nerve system and the lipopolysaccharides (LPSs) of Campylobacter jejuni (Houliston et al., 2011; Yuki et al., 2004). A new hypothesis proposed that infectious agent can trigger an upregulation of the host immune response to self-antigens, which may cause autoimmune diseases (Yanagisawa et al., 2011). The regression of autoimmune thrombocytopenia after the eradication of Helicobacter pylori is supporting this theory (Jackson et al., 2005). However, the genetic contribution to autoimmune pancreatitis (AIP) remains elusive.

1.2.1.6 Genetics of CP and AIP

Recently the genetical basis of CP has been extensively studied. In this regard, Whitcomb et al. were the first who reported a mutation in the cationic trypsinogen gene (*PRSS1*), *p.R122H*, resulting in hereditary pancreatitis (Whitcomb et al., 1996). This gain-of-function mutation stabilises activated trypsin by preventing its autolysis. Lately, more CP associated genes have been identified, and specifically genes that are known to be contributing factors rather than causative genes. Genes such as *SPINK1* (Serine Protease Inhibitor Kazal type1) (Witt et al., 2000), *CFTR* (Cystic Fibrosis Transmembrane Regulator; the gene which is mutated in cystic fibrosis) and *CTRC* (Chymotrypsinogen C) are considered to be the best characterised susceptibility genes (Rosendahl et al., 2007; Sharer et al., 1998). Intriguingly, in

all above-mentioned genes, a loss of function has been reported following mutations in CP patients. In a study by Witt et al., a variation has been identified in the anionic trypsinogen gene *PRSS2* that serves as a preventing factor against CP (Witt et al., 2006).

The genetic basis of AIP has not been systematically investigated so far. As reported by Ota et al., two critical genes (i.e. *HLA-DRB1* and *ABCF1*) in the HLA region were found to be related to the susceptibility to AIP (Ota et al., 2007). Interestingly, the human risk allele *HLA-DRB1*0405* predisposes class II transgenic Ab0 NOD mice to AIP (Freitag et al., 2010). Similar to patients with other autoimmune diseases, AIP patients have a particular HLA haplotype (i.e. *DRB1*0405-DQB1*0401*) (Guarneri et al., 2005; Klöppel et al., 2003) that serves as an important risk factor for AIP. Recently, two other risk factors have been reported for AIP: human Carbonic anhydrase II (*CA-II*) and α-Carbonic anhydrase of Helicobacter pylori (*HpCA*) that are significantly homologous and share segments that contain the binding motifs of the HLA molecule *DRB1*0405* (Klöppel et al., 2003). Additionally, a mutation of *DQ beta1* (substitution of aspartic acid at position 57) was identified as an important genetic factor for relapse of AIP (Park, Kim et al., 2008). Previous reports show that polymorphisms of the Cytotoxic T-lymphocyte antigen 4 (*CTLA-4*) gene have been linked to the disease in Chinese and Japanese populations (Chang et al., 2007; Umemura et al., 2008). In 2013, an association between mutations in the protease serine 1 (*PRSS1*) gene and AIP has been reported for the first time (Gao et al., 2013). Furthermore, an association between mutations in the *KRAS* gene and pancreatobiliary carcinoma has been recently reported in patients with AIP (Ohtani et al., 2011).

1.2.1.7 Mouse Models of AIP

Pathological mechanisms and genetic factors regulating diseases can be studied using animal models of human diseases due to their genetical, anatomical and physiological similarities to humans. In addition, their unlimited supply, ease of manipulation, high reproductive rates and relative low cost of use make them valuable for the majority of genetic diseases studies. Over the last decade, our understanding of the cause and progression of human genetic diseases based on animal models studies have greatly improved. Furthermore, animal models have proven to be a powerful tool for the discovery of novel therapeutic targets (Simmons, 2008). Within animal models, mice are the predominantly used laboratory animals for studying the etiology and the pathogenesis of autoimmune diseases. For example, the alylaly (*aly-/aly-*) mouse is a unique model of spontaneous Sjögren's syndrome (Tsubata et al.,

1996). As shown in Table 1.2, mice, rat or other species are useful tool for investigation of the pathoetiology of autoimmune diseases.

Recent development of AIP animal models enhanced the understanding of the pathogenesis of the disease, which is a newly described form of chronic pancreatitis (Park et al., 2009). Several murine models have been employed to understand the cellular and genetic mechanisms involved in the development of AIP. For example, Davidson et al. reported that the administration of activated $CD4^+$ T cells[3] specific for the pancreatic enzyme amylase can induce pancreatitis in the experimental autoimmune pancreatitis models "DA(PR) rats" and "Lewis rats" (Davidson et al., 2005). Other models include the WBN/Kob rats that are used to study AIP with autoimmune extrapancreatic exocrinopathy and which show several features of the human condition, i.e. infiltration of $CD8^+$ cells, deposition of tissue-specific IgG2b in the injured pancreas/lacrimal glands and decreased number of regulatory T cells (defined as $CD4^+$ $FoxP3^+$ cells)[4] in the periphery of both sexes. Altogether, these results suggest that WBN/Kob rats represent a novel model for the study of AIP, Sjögren-like syndrome or multi-focal fibrosclerosis in humans (Sakaguchi et al., 2008). Animal experimental models of autoimmune pancreatitis are listed in Table 1.2.

The MRL strain is a well-known mouse model for AIP that was developed from several crosses of inbred strains. Theofilopoulos & Dixon `s showed that most of the MRL genome was derived from the LG/J strain with minor contributions from C3H/Di, C57BL/6, and AKR/J (Theofilopoulos & Dixon, 1984). The substrain MRL/lpr developed an SLE-like phenotype characterized by lymphadenopathy due to the accumulation of double negative ($CD4^-CD8^-$) and $B220^+$ T-cells. These mice showed an accelerated mortality and both males and females were significantly affected. Furthermore, high concentrations of circulating immunoglobulins including elevated levels of autoantibodies such as Anti-nuclear antibody (ANA), Anti-single stranded DNA (anti-ssDNA), Anti-double stranded DNA (anti-dsDNA), Anti-Smith (anti-Sm) and Rheumatoid factors (RF) are detected in MRL/lpr substrain, which may lead to formation of immune complexes (Andrews et al., 1978; Perry et al., 2011).

Previously, it has been shown that aged MRL/Mp-+/+(MRL/+) mice develop spontaneously an autoimmune pancreatitis. Several studies proposed that polyinosinic:polycytidylic acid (poly I:C), a synthetic analog of double-stranded RNA,

[3] CD stand for cluster of diffrentiation
[4] Forkhead-Box-Protein P3

triggers the development of autoimmune diseases including pancreatitis in a particular genetic background (Okada et al., 2005; Qu et al., 2002). Autoimmune pancreatitis, as a multi-factorial disease, can be controlled by genetic and environmental factors. Therefore, MRL mice treated with polyinosinic:polycytidylic acid (poly I:C) might be a useful mouse model to study the pathogenesis of AIP (Qu et al., 2002). Using AlP-prone MRL/Mp mice, Fitzner et al showed that interferon gamma (IFN-γ), a T helper1 (Th1) pro-inflammatory cytokine, plays a key role in the inflammatory process. Therefore, they proposed that IFN-γ-enhanced AIP in MRL/Mp mice and can be useful to study the pathophysiology, diagnosis and therapeutic approaches for AIP (Fitzner et al., 2009).

Recent studies showed that AIP-like diseases could be induced in most animal models by exposure to heat-killed *E. Coli* or adoptive transfer of autoreactive cells and self-antigens such as carbonic anhydrase II (CA-II) and lactoferrin (LF) (Haruta et al., 2010; Okazaki et al., 2010). AIP-like pathological alterations were observed in pancreas and salivary glands of some mice models of AID (C57BL/6 and BALB/c) after repeated exposure to heat-killed *E. Coli* (Yanagisawa et al., 2011). AIP is induced when genetic factors such as haplotype of class II antigen of the major histocompatibility complex (MHC) are synchronized with the presence of commensal bacteria or pathogenic microorganisms (Haruta et al., 2012).

The limited availability of animal model for research has slowed down the related studies on the pathophysiology and genetics of AIP. In the current study, a MRL/Mp mouse model (established by Kanno et al.) was chosen for three main reasons. First, it closely reflects the histopathological changes typical to human AIP. Second, the disease develops spontaneously (Kanno et al., 1992; Sorg et al., 2008) and may be further aggravated by trigger substances such as polyinosinic:polycytidylic acid (Qu et al., 2002) or IFN-γ (Fitzner et al., 2009). Finally, its autoimmune pathogenesis, which was supported by induction of pancreatitis (in females) following adoptive transfer of splenic cells obtained from diseased mice (Kanno et al., 1992). It has been shown that MRL/Mp female mice exhibit early onset and higher severity of AIP as compared to males (Kanno et al., 1992; Sorg et al., 2008). The murine disease cannot be simply classified according to the human subtype system due to the lack of IgG4 in rodent species. However, some similarities exist between the AIP of MRL/Mp mice and human AIP subtype 1. For instance, the lack of typical granulocytic epithelial lesions, and the absence of non-specific autoantibodies (Kanno et al., 1992; Sorg et al., 2008).

Table 1.2 Summary of animal experimental models of autoimmune pancreatitis

Authors	Year	Animals	Target antigens	Induction	Organs with lesions	Effector cells
Kanno et al.	1992	MRL/lpr mice	?*	Spontaneous	Pancreas	T cells
Nishimori et al.	1995	PL/J mice	CA-II	Subcutaneously immunised with CA-II	Salivary	?
Tsubata et al.	1996	aly⁻/aly⁻mice	?	Spontaneous	Pancreas, Peyer's patch deficiency	$CD4^+$ T cells
Haneji et al.	1996	nTx-NFS/sld mice	a-fodrin	Spontaneous	Salivary	$CD4^+$ T cells
Uchida et al.	1998	nTx-BALB/c mice, and nude mice	CA-II	Subcutaneously immunised with CA-II	Pancreas, bile duct, salivary glands , renal tubule	$CD4^+$ Th1 cells
Uchida et al.	1998	nTx-BALB/c and nude mice	LF	Subcutaneously immunised with LF	Pancreas, bile duct, salivary glands, renal tubule	$CD4^+$Th1 cells
Vallance et al.	1998	MHC-class II-deficient mice	?	?	Pancreas, colon	$CD8^+$ T cells
Ueno et al.	1998	BALB/c mice	CA-II	Intraperitoneally immunised with CA-II	Bile duct	$CD4^+$ T cells
Mustafa et al.	1998	MRL/lpr mice	?	?	Salivary	Th1
Uchida et al.	2002	nTx-BALB/c mice and nude mice	CA-II, LF	Subcutaneously immunised with CA-II, LF	Pancreas, bile duct, salivary gland, renal tubule	$CD4^+$ Th1 cells
QU et al.	2002	MRL/+, MRL/lpr	?	poly I:C	salivary, bile duct, renal tubule	$CD4^+$ T cells
Watanabe et al.	2003	C57BL/6 mice	?	LP-BM5	Pancreas, salivary gland, liver, kidney, lung	$CD4^+$ T cells
Davidson et al.	2005	DA(PR) rats and Lewis rats	amylase ?	Amylase-specific T cell	Pancreas	$CD4^+$ T & $CD8^+$ T cells
Sakaguchi et al.	2008	WBN/Kob rats (Wistar Bonn Kobori rat)	?	Spontaneous	Pancreas and lachrymal gland	$CD8^+$ T cells
Meagher et al.	2008	NOD.CD28KO mice	Amylase	Spontaneous	Pancreas	$CD4^+$ T cells
Haruta et al.	2010	C57BL/6 mice	?	E. coli	Pancreas, salivary gland	T cells

CA-II, carbonic anhydrase II; CD; cluster of differentiation, LF, lactoferrin; nTx, neonatal thymectomy; LP-BM5 murine leukemia virus; Th1, T helper 1; poly I:C, polyinosinic:polycytidylic acid.

*? = There is no information available and target remains to be explored.

Adopted from (Okazaki et al., 2001; Okazaki et al., 2008; Park et al., 2009).

1.3 Strategies to Identify Susceptibility Genes of Complex Diseases

Everybody has a different genome due to the gene variations, which contribute to most of the complex diseases. Therefore, any attempt to identify susceptibility genes for a particular disease improves our knowledge of the molecular mechanisms, diagnosis and prevention of the disease, as well as finding new targets for the development of therapeutic strategies. Many interacting genes and environmental factors affect complex diseases and each of these factors may play a role, which can have an effect on the disease in a certain genetic and environmental background (Lander & Schork, 1994). Because of this complexity, large source of families involving several affected individuals are required to reach significant result in human linkage analysis. Using inbred mice, which are genetically homogenous, could be an alternative for this purpose. Inbred mouse strains with various genome backgrounds will show variant susceptibility to a particular disease that resembles human disease. Crossing between the susceptible strain and the resistant strain could be helpful in identifying Quantitative Trait Loci (QTLs) affecting the susceptibility to the disease as well as genes underlying susceptibility to human disease. Human studies of prone gene can be carried out as soon as the susceptible gene is identified in mice.

1.3.1 From Disease to Quantitative Trait Loci by Linkage Analysis

To date, different strategies are developed to identify QTLs. Within these strategies, the location of genes that cause genetic diseases could be essentially identified by linkage mapping. Therefore, the first breeding step performed in a linkage analysis is an outcross between two inbred strains or between one inbred strain and a non-inbred animal that contains a genetic variant. The main types of crosses that are usually used in mouse genetics are N2 backcrosses and F2 intercrosses, which are obtained from F1 mice intercrossing. N2 backcross and F2 intercross are commonly used for the linkage analysis to identify QTLs in selected susceptible and resistant strain (Abiola O et al., 2003). To produce F2 intercross mice, two parental strains that have the same heterozygous genotype at designated loci are crossed to produce F1 mice. Then these F1 mice are intercrossed to produce next generation (F2). In contrast, N2 backcrosses are obtained from crossing between F1 mice (heterozygous for alleles obtained from two parental strains) and one of those parental strains. Therefore, F2 intercrosses have been chosen for linkage analysis (due to the dual recombination events in the same number of mice) rather than N2 backcrosses. Two examples in this filed are identification of QTLs controlling autoimmune diabetes in NOD mice and genetic linkage

analysis of collagen-induced arthritis in F2 intercross mice (Jirholt et al., 1998; Wicker et al., 1995).

1.3.2 QTLs Mapping

Quantitative trait loci (QTLs) are stretches of genome that contain or are linked to one or more susceptibility genes, which are controlling a quantitative trait. Mapping the QTLs is done using molecular tags such as Amplified fragment length polymorphism (AFLP) or more commonly, single nucleotide polymorphisms (SNPs). Quantitative traits refer to phenotypes that differ in degree and can be associated with polygenic effects, i.e., product of two or more genes, and their environment. However, it remains challenging to identify and map QTLs considering many genes that located inside this genomic region, possible epistasis or interactions between QTL and many additional sources of variation (Doerge, 2002). In an attempt to increase mapping resolutions (fine mapping of QTL), narrowing the confidential interval (CI) is required. The main goal of fine mapping of QTL is to identify candidate genes within a certain region (<1~5 cM or ~ 1-2 Mbp) of the genome. Several approaches were proposed for fine-mapping of QTLs, i.e. Advanced intercross line (AIL), subcongenic strains, partial advanced intercross lines (PAIL), haplotype analysis, Yin-Yang crosses, outbreed stocks, and recombination inbred lines (RIL) (Flint, 2011; Flint et al., 2005).

An Advanced Intercross Line (AIL) is originated from two parental inbred strains by intercrossing and followed by subsequently and randomly intercrossing, avoiding brother-sister mating, for several generation. This approach provided animals, which accumulated many recombination events (Yu et al., 2007), which can be useful for QTLs identification. The first step begins with crossing of two parental strains to produce F1 mice. The procedure continues by intercrossing these F1 mice to produce F2. To produce following generations, randomly intercrossing within each generation is performed via at least 50 random mating per generation. Sufficient random mating in each generation provide the heterogeneity of all the alleles in the genome, which makes AIL valuable for fine mapping of all the QTLs identified in the mice. However, the obvious limitations of such approach are time and space that are required to produce the experimental animals.

Subcongenic Strain is another commonly used fine-mapping approach. These strains are produced from congenic strains containing the QTLs genome intervals. A set of subcongenic strains have a short differential segment and recombination events in different

locations of the genome. Consequently, QTLs can be distinguished by evaluation of a set of these strains. The refining size of a QTL is dependent on overlapping and the number of subcongenic strains (Fehr et al., 2002). Additionally, as subcongenic strains are genetically identical mice, they can be used to detect and refine the QTLs that have only small effects. However, besides being time consuming, other inadequacies of this approach are again the large space and workload needed.

Partial Advanced Intercross Lines (PAIL) have been developed by combining the advantages of both AIL and subcongenic strains. PAIL mice are generated using a series of intercrosses between congenic mice and the wild-type mice as parental strains (Johannesson et al., 2005). With identical genetic background and high recombination events, PAIL mice can be used to refine a small effect QTL into a rather small genome region. However, PAIL also has the shortcomings of AIL and subcongenic strains, i.e. being time and space consuming.

Haplotype analysis might be the fastest method for QTLs fine-mapping, which reduced the required time designed for genetic analysis for complex diseases from months to seconds (Grupe et al., 2001). The method is based on identification of a QTL in different inbred-strain crosses following by combination of the strain distribution pattern with mapping data, to refine the region that contains the functional variant (Flint et al., 2005).

Yin-Yang crosses, outbred stocks, and recombination inbred lines (RIL) are some other approaches recommended for QTLs fine-mapping (Flint et al., 2005). It is also important to keep in mind that each approach has own advantages and disadvantages. Therefore, the choice of method for fine mapping of QTLs is dependent on particular phenotypes or QTLs rather than method itself.

1.3.3 Identification of Susceptibility QTL of AIP in Four-way AIL

Because of accumulated numerous combination events, AIL is a powerful approach for high-resolution fine mapping of QTLs compared with conventional F2 intercross N2 backcross, (Yu et al., 2006). Therefore, a four-ways advanced intercross line (4-way AIL) could provide much more detailed approach to identify and refine the regions (QTLs) that are contributing to certain diseases.

1.4 Aims of This Study

The main three aims that underlie this thesis are:

- Identification of genes contributing to autoimmune diseases susceptibility,
- Identification of novel QTLs that control the susceptibility to AIP,
- Investigation of immunopathogenesis of AIP in the four-way AIL animal model of AIP.

To address the first aim of the thesis and to identify susceptible genes to autoimmunity we had generated an outbred intercross mouse line, a four-way autoimmune-prone advanced intercross line (AIL), originating from MRL/MpJ (spontaneous autoimmune pancreatitis), BXD2/TyJ (spontaneous arthritis), NZM2410/J (spontaneous Lupus) and Cast/EiJ mice (wild derived strain). The progeny of this cross represents a large family with higher incidence of common autoimmune diseases such as arthritis, lupus and AIP. Next, a classical quantitative trait mapping approach was performed to identify novel loci controlling susceptibility to AIP. Finally to study the immunopathogenesis of AIP, the role of regulatory T cells (Tregs) and T helper cells was addressed using the four-way AIL mouse model.

2 Materials and Methods

2.1 Materials

2.1.1 Instrument & Laboratory supplies

Centrifuge	Eppendorf, Germany
Centrifuge (Rontina 464)	Hettich, USA
LABOFUGE centrifuge 400R	Thermo Scientific
Microcentrifuge, mikro 200R	Beckman Coulter, Germany
BD FACS calibor	Becton Dickinson (BD), USA
Precision pipette set (10/20/100/200/1000 µl)	Eppendorf, Germany
Sterile Hood (laminarair @ HB2448)	Heraeus Instrument, Germany
Light microscope	Zeiss, Oberkochen, Germany
Light microscope	Leica, Germany
Zeiss axiophot microscope	Axiophot, Zeiss, Oberkochen, Germany
Microtome	Leica CM1850, Germany
Microtiter plates (96well, round bottom)	Nunc, Germany
Cryo pure tube	Sarstedt, Germany
Cell strainer 70	Becton Dickinson(BD), USA
Precision pipet set (10, 20, 100, 200 &1000 µl)	Eppendorf, Germany
Round-bottom FACS tubes (5 ml)	Becton Dickinson, USA

2.1.2 Commercial kits

QIAamp® DNA Blood Mini Kit	QIAGEN, Hilden, Germany
QIAamp® DNA Mini Kit	QIAGEN, Germany
ABC staining kit	Vector Laboratories, Burlingame, California, USA
DNeasy Blood & Tissue Kit	QIAGEN, Germany
RNeasy Mini Kit	QIAGEN, Germany
RNAlater	Ambion, Germany

2.1.3 Chemical and Reagents

NaCl	Roth, Germany
KCl	Merck, Germany
$Na_2HPO_4.2H_2O$	Merck ,Germany
KH_2PO_4	Merck, Germany
Tris-HCl	Roth, Germany
EDTA[5]	Roth, Germany
PBS[6] (10X)	Gibco, Life Technologies, USA
FCS[7]	PAA, GE Healthcare, Germany
RPMI 1640 medium	PAA Laboratories, Germany
NaOH	Merck, Germany
Trypanblue	Biochrom, Germany
Penicillin/Streptomycin	PAA Laboratories, Germany
D-MEM Medium	GIBCO®, USA
Fetal calf serum (FCS)	GIBCO®, USA
Penicillin/Streptomycin	GIBCO®, USA
RPMI 1640 Medium	GIBCO® ,USA
Trypsin-EDTA (1x)	GIBCO®, USA
Tissue-Tek® O.C.T™ Compound	Sakura Finetek Europe B.V, The Netherlands
Erythrocyte lysis buffer was obtained from	QIAGEN®, Germany
1X Red Blood Cell Lysis Buffer	eBioscience, USA
PBS	Gibco, USA
Formafix 4%	Grimm med. Logistik GmbH; Germany
Paraffin	Roth, Germany
Paraformaldehyd	Morphisto, Germany

2.1.4 Antibodies

FITC anti-mouse CD4	BD PharMingen™, USA
PE anti-mouse B220	BD PharMingen™, USA

[5] Ethylenediaminetetraacetic acid
[6] Phosphate buffered saline
[7] Fetal calf serum

PerCP-Cy5.5 anti-mouse CD8	BD PharMingen™, USA
APC anti-mouse CD3	BD PharMingen™, USA
FITC anti-mouse CD44	BD PharMingen™, USA
PE anti-mouse CD62L	BD PharMingen™, USA
PerCP-Cy5.5 anti-mouse CD4	BD PharMingen™, USA
APC anti-mouse CD69	BD PharMingen™, USA
FITC anti-mouse CD86	BD PharMingen™, USA
PE anti-mouse MHC Class II	eBioscience, USA
PerCP-Cy5.5 anti-mouse CD19	BD PharMingen™, USA
PE anti-mouse PDCA-1	eBioscience, USA
APC anti-mouse CD11c	BD PharMingen™, USA
PE anti-mouse IL17*	BD PharMingen™, USA
APC anti-mouse CD8	BD PharMingen™, USA
PE anti-mouse FoxP3	eBioscience, USA
FITC anti-mouse IFN gamma*	BD PharMingen™, USA
APC anti-mouse IL4*	BD PharMingenTM, USA
FITC anti-mouse CD25	BD PharMingen™, USA
PE Armenian Hamster IgG Isotype Control	eBioscience, USA
Anti-CD4	Immunotools, Friesoythe, Germany
Anti-CD45R (B220)	Immunotools, Friesoythe, Germany
Anti-CD8	Santa Cruz Biotechnologies Inc., USA
Anti-F4/80	eBioscience, San Diego, USA

*These antibodies target the intracellular (IC) antigens. PE; Phycoerythrin, APC; Alkaline Phosphate Conjugated, FITC; Fluorescein Isothiocyanate conjugated, (PerCP)-Cy5.5; Peridinin chlorophyll protein -*Cy5.5*.

2.1.5 Buffers and Solutions

10 x PBS Buffer:

NaCl	1.37 M	80 g
KCl	27 mM	2.0 g
Na_2HPO_4. $2H_2O$	80 mM	14.4 g
KH_2PO_4	17.6 mM	2.4 g

Dissolve in 800 ml dH_2O, adjust the pH to 7.4, and add dH_2O until a total volume of 1 liter and autoclave.

FACS washing buffer

1 x PBS, 0.04% NaN_3, 5% FCS

FACS-Wash Buffer (Staining Buffer):

1 X PBS

3% heat-inactivated FCS

FCS buffer adjust pH to 7.4-7.6

Filter (0.2 µm pore membrane), and store at 4 ºC

Fc Block:

Dilute anti-CD16/CD32 (0.5 mg/ml)
Add 120 µl Fc-block + 1080 µl of FWB dilution factor 1/10

TE Buffer

Tris-HCl 10 mM, EDTA1 mM,
Adjust the pH to 8.0

2.1.6 Mice

All mice used in this study were housed under climate-controlled environment with 12-h light/darkness cycle with food and water ad libitum in the animal facility at the University of Rostock. The procedures, assays and all animal studies were pre-approved by local governmental authority, i.e. the Animal Care Committee of Mecklenburg-Vorpommern. The parental strains MRL/MpJ, NZM2410/J, BXD2/TyJ and Cast/EiJ were purchased from Charles River Laboratories (Sulzfeld, Germany).

2.1.6.1 Generation of Four-way Autoimmune Advanced Intercross Line (AIL)

In this study, an outbred four-way autoimmune prone advanced intercross line (AIL) was originated from the parental mouse strains MRL/MpJ and NZM2410/J, BXD2/TyJ and Cast/EiJ (Figure 2.1). Parental MRL/MpJ mice are prone to develop spontaneous AIP as previously described, in an age and gender-specific manner, whereas the other strains displayed no significant histopathological changes of the pancreas. The lupus-prone NZM2410/J strain was originated from NZB × NZW mice. The BXD2/TyJ mice are particularly well known for developing spontaneous arthritis and generalized autoimmune disease. In contrast, wild type strain Cast/EiJ is resistant to these diseases. The four parental

strains were intercrossed at an equal strain and sex distribution in order to maintain an equal distribution of the original strains in the following generations. The parental origin of generation 1 (G1) offspring mice was considered when setting up mating for the generation of G2 mice. This procedure was also followed for intercrossing G2 mice. At each time, at least 50 breeding pairs were used as parents for the next generation of mice. In this work a quantitative trait mapping (QTL mapping) analysis was performed to identify quantitative trait loci associated with AIP in G4 mice.

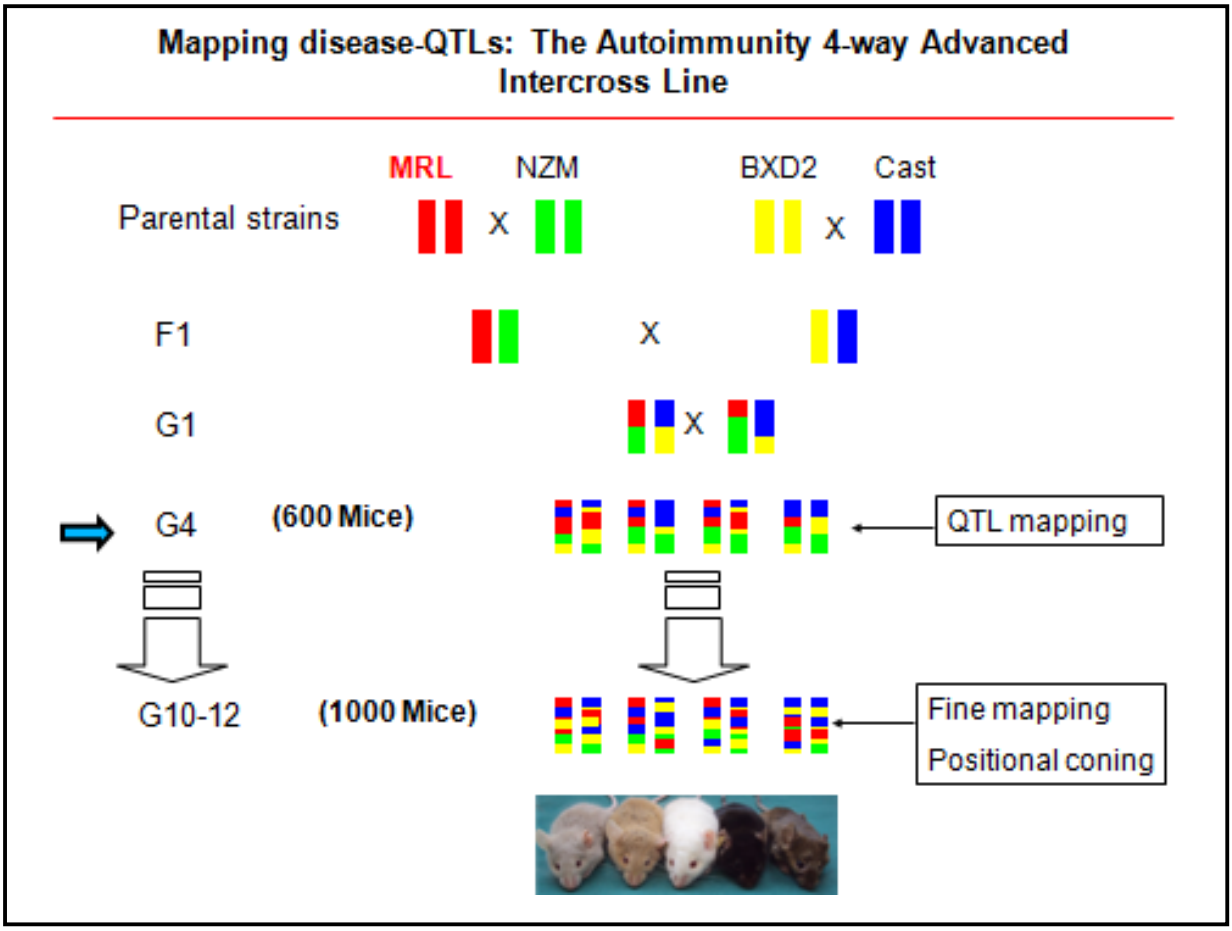

Figure 2.1 Schematic representation for mapping disease-QTLs: the autoimmunity four-way advanced intercross line (courtesy of Prof. Saleh Ibrahim)

2.1.6.2 The Fourth Generation (G4) of Four-way AIL Mice

A total of 351 mice from fourth generation (G4) of the four-way autoimmune-prone AIL were maintained in animal facility under specific pathogen-free conditions and monitored for clinical status and development of autoimmune diseases. In a joint work within our group, mice were assessed twice per week for onset, joint inflammation, skin disorders and any

abnormality. Six-month-old mice were sacrificed and samples were obtained for further analysis. The development of spontaneous AIP was assessed in parental strains and in the fourth generation (G4) of four-way advanced intercross line by scoring the severity of pancreatic lesions as described in section (2.2.1.2.1). Mice were kept under specific pathogen free conditions as mentioned above.

2.2 Methods

2.2.1 Protocol for AIP and Autoimmune Diseases Studies

2.2.1.1 Sacrificing and Sampling

For this study, six-months-old G4 mice of four-way advanced intercross line were subjected to the experiments according to the following protocols:

- The mice were anaesthetized using diethyl ether and sacrificed by cervical dislocation. Weights of the mice were measured at autopsy.
- Blood was collected and sera were obtained after centrifugation at 14000 rpm for 10 min, aliquoted (100 µ*l* in each tube) and stored at -20 °C then at -80 ºC for further purposes (to assay autoantibodies using proteome arrays or ELISA, as well as to assay BUN, lipase, glucose and so on).
- 1 cm-piece of mouse-tail tip was cut and placed in a 1.5 ml micro-tube on ice, then stored at -20 ºC for DNA isolation.
- Abdomen was opened; pancreas, spleen, kidney, lymph nodes, liver and lung were taken for histopathological examination and other purposes.

Pancreas

Dissected pancreas was divided into 3 parts:

- Left part (next to duodenum) was fixed in 4% phosphate buffered formalin for 3 days. The tissues were then embedded in paraffin. Serial sections (5 µm) were used for histopathologic evaluation of pancreatic lesions after staining with haematoxylin and eosin (H&E).
- The middle part has been quickly frozen in liquid nitrogen and stored at -80 ºC.

- The right part was placed in OCT, and then it has been frozen in liquid nitrogen and stored at -80 ºC until use. The 6 µm serial sections of this part of pancreas were stained for immunohistochemical studies.

Spleen

Weight of each dissected spleen was measured before it was divided it into 3 pieces:

- One piece in RPMI (with 10% FCS) for following steps:
  - to prepare splenocytes suspension,
  - to count and to adjust the cell density to 10^6 cells in 10 µl of cell suspension,
  - To examine cell population/ subsets and activation, multicolour flow cytometric analyses were performed with antibody cocktails, i.e. B220, CD3, CD4, CD8, CD62 L, CD86, CD69, CD11c immediately after removing the spleen.
- The second piece was placed in liquid nitrogen and stored at -80 ºC until use (for signaling proteome arrays, were shipped on dry ice for processing and analysis).
- Finally, the last piece was placed in OCT, and has been frozen in liquid nitrogen and stored at -80 ºC (for subsequent pathology scores and antigen specific immunohistochemistry).

Kidney

- Left kidney was placed in formalin overnight and later embedded in paraffin.
- In addition, the right kidney was divided into two parts; one part was placed in OCT and then frozen at -80 ºC. The other part of kidney was placed in liquid nitrogen and then stored at -80 ºC (for signalling proteome arrays).

Lymph nodes

Two lymph nodes (cervical or inguinal) were dissected and then placed into RNA later. The samples were incubated at 2-8 °C overnight, and were then archived at -20 °C for future studies.

Lung & liver

Dissected pieces of lung and liver were placed into OCT and has been frozen in liquid nitrogen and then stored at -80 ºC.

2.2.1.2 Spontaneous AIP

Development of spontaneous AIP in parental strains (54mice) and fourth generation of intercross mice (G4) (163 males and 188 females) was assessed in 6-month-old mice by scoring the severity of pancreatic lesions as outlined below.

2.2.1.2.1 Histology

Half of the pancreas of each mouse was fixed in 4% phosphate buffered formalin and then embedded in paraffin. From paraffin-embedded pancreatic blocks, 5 μm thick sections were prepared and stained with Haematoxylin and Eosin (H&E), applying standard protocols. The histopathological evaluation of pancreatic lesions was performed employing a light microscope. Pathological changes in each sample were scored on a semi-quantitative scale from 0 (healthy pancreas) to 4 (severe AIP) grade, using an established scoring system with minor modifications (Qu et al., 2002; Sorg et al., 2008). Briefly, severity of pancreatic lesions was quantified as following:

- Stage zero (0), no pathological changes; pancreas without mononuclear cell infiltration, indicating almost normal tissue morphology and organ integrity;
- Stage one (1), minimal infiltration of periductal tissue with mononuclear cells but no parenchymal destruction; mononuclear cell aggregation and/ or infiltration within the interstitium without any parenchymal destruction;
- Stage two (2), moderate periductal infiltration with mononuclear cells associated with beginning parenchymal destruction; focal parenchymal destruction with mononuclear cell infiltration;
- Stage three (3), severe periductal inflammation and/ or more extended parenchymal destruction; diffuse parenchymal destruction but retained some intact parenchymal residue;
- Stage four (4), diffuse mononuclear cell infiltration in pancreatic tissue except of pancreatic islets, destruction of acini and (partial) replacement by adipose tissue.

All samples were blinded before evaluation. To estimate the incidence of pancreatitis in G4 mice, pancreatic lesions that scored ≥ 2 were defined as positive for AIP. The slides were reviewed and scored by Prof. Horst Nizze as a qualified pathologist and Prof. Robert Jaster.

2.2.1.2.2 Immunohistochemistry

The other half of the pancreas was embedded in OCT compound (Sakura Finetek Europe B.V, The Netherlands) and then frozen in liquid nitrogen and stored at -80 °C. An immunohistochemical (IHC) staining was performed using antibodies specific for CD4, CD8, CD45R (B220) and CD138. Six mm thick serially cut sections were fixed by incubation in ice-cold methanol for 1 min at 4 °C. Subsequently, sections were washed three times with phosphate buffered saline (PBS), and stained using the VECTASTAIN ABC staining kit (Vector Laboratories, Burlingame, California, USA) according to the manufacturer's instructions (Figure 2.2). The following mouse specific primary rat antibodies were used for the characterization of leukocyte subsets: anti-CD4 (Immunotools, Friesoythe, Germany), anti-CD8 (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), anti-CD45R (B220) (Immunotools, Friesoythe, Germany), and anti-CD138 (eBioscience, San Diego, California, USA). All primary antibodies were yielded in rats, directed against the relevant murine antigen and used at a 1:100 dilution. The sections were counterstained with Haematoxylin and examined by light microscopy (Axioskop 40, Zeiss, Oberkochen, Germany).

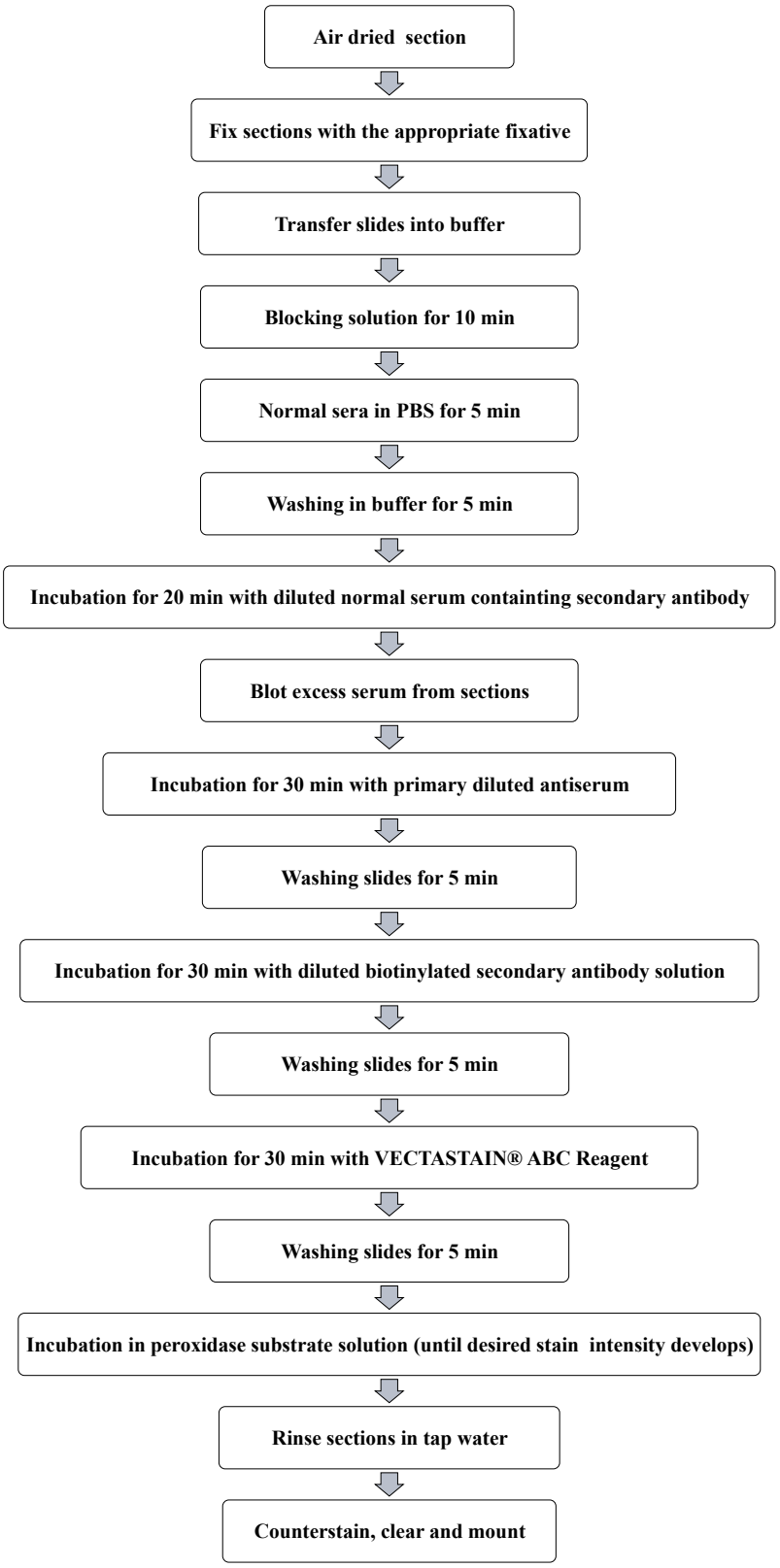

Figure 2.2 Schematic representation of immunohistochemical staining protocol performed on frozen sections.

Based on VECTASTAIN ABC staining kit protocol (Vector Laboratories, Burlingame, California, USA). (http://www.vectorlabs.com/data/protocols/PK-4000.pdf)

2.2.2 Molecular Biological Methods

2.2.2.1 Isolation of Mouse Total DNA

Genomic DNA was isolated according to the following protocol: mouse-tail tips (0.5-0.8 cm) were incubated with 500 µl 50 mM NaOH for 2 hours at 95 °C. In order to make uniform solution, the samples were mixed by occasional vortexing during incubation period. DNA neutralization was done by adding 50 µl of 1 M Tris-HCl (pH 8.0) in each tube. Afterwards, 500 µl double-distilled water (dd H_2O) was added to the tube and followed by centrifugation at 10,000 g for 5 min. Finally, the supernatant was discarded and the pellets were dissolved as total mouse DNA. The DNA samples were then stored at -20 ^{o}C.

For genotyping using Illumina murine High Density array (Illumina, California, USA), pure genomic DNA was isolated with the DNeasy Blood & Tissue Kit according to manufacturer's instructions. Briefly, a 0.5 cm mouse-tail tip was incubated with 180 µl buffer ATL and 20 µl Proteinase K at 56 °C until the tissue was completely lysed. Then 20 µl RNase-A was added to eliminate the RNA from sample. In the next step, the sample was incubated with 200 µl buffer AL for 10 min at 70 °C, then 200 µl ethanol were added and the sample was applied to the QIAamp spin column. Finally, the DNA was eluted by using AE buffer after washing with AW1 and AW2 buffers.

2.2.2.2 Quantitative Trait Loci (QTLs) Identification

To identify quantitative trait loci (QTLs) associated with autoimmune diseases, we established an advanced intercross line originating from MRL/MpJ parental mice and the following three mouse strains: Cast (healthy controls), BXD2 (susceptible to collagen-induced arthritis) and NZM (a model of Lupus erythematosus). This concept was chosen to identify both general autoimmune disease-associated loci and AIP-specific QTL. Therefore, the fourth generation (G4) of outbred intercross mice was characterized phenotypically by scoring histopathological changes of the pancreas and genotyped with SNP arrays (Illumina, USA).

2.2.2.3 QTL Mapping

A total of 330 mice of the fourth generation were genotyped by a set of 1400 single nucleotide polymorphisms (SNPs) using an Illumina murine HD array (Illumina, California, USA). Genotype/ phenotype correlation was performed with the R version of HAPPY (Mott

et al., 2000) in version 2.3 beta on Debian Linux in collaboration with Dr. Steffen Möller (Möller et al., 2010). The data from all chromosomes were analysed together with an additive model and their log P value and tested positively against 1000 permutations for pointwise significance ($P < 0.001$). The flanking regions of QTLs were determined manually by a drop of 1.5 to both sides to the peak.

2.2.3 Immunological Methods

2.2.3.1 Flow Cytometry

2.2.3.1.1 Cell Surface Markers Staining

Single cell suspensions preparation: In this study, to analyse subtypes of T cells, B cells and dendritic cells, single-cell suspensions from spleen of G4 mice were prepared as following: One third of mouse spleen was excised, suspended in 5 ml RPMI-1640, dissociated by using a 70 µm nylon cell strainer and washed twice in PBS. The cells were pelleted by centrifugation at 1200 rpm for 5 min at 4 °C. The supernatant was discarded and the pellet resuspended in 3 ml of 1 X RBC (red blood cell) lysis buffer. Then, the tubes were incubated on ice for 4-5 minutes with occasional shaking. The reaction was stopped by diluting the lysis buffer with 5 ml of RPMI. The cells were centrifuged (at 1200 rpm for 5 min), then washed with 10 ml medium to remove lysis buffer (2 X wash). The cells were spin at 1200 rpm for 5 min at 4 °C and then resuspended cells in 1 ml medium. Cell numbers were counted and viability analyses were done (10 µl cell + 50 µl Trepan Blue + 40 µl FWB, dilution factor 1/10). Volumes were corrected to 100 million cells/ml. Then, 10 µl of adjusted cell suspensions (equal to 10^6 cells) were added to each well for surface marker staining. For intracellular staining, 12 µl of cell suspensions were added to each well. In addition, a second round of lyses was performed for some samples with a small number of residual red cells.

Block Fc Receptors step: Cells were pre-incubated with 2 µl anti-CD16/CD32 (dilution factor 1/10) per million cells for 5-10 min on ice prior to staining.

Antibody preparation and incubation: The cocktails of antibodies were prepared and diluted to previously determined optimal concentration of primary antibody in staining buffer (Table 2.1). 2 µl of antibody-cocktails were dispensed to each well in 96-well plate. Two µl of staining buffer was added to each unstained or negative control wells and mixed gently. The 20 min incubation in dark on ice was performed for all samples. Dependent on our

preliminary experiments, for some antibodies incubation times were longer. Following the incubation period, two washing steps were performed by adding 200 µl of FACS wash buffer (FWB). Cells were centrifuged at 2000 rpm for 2 min at 4 °C and the supernatant was discarded. The stained cells were fixed with 1% paraformaldehyde for 10-20 min at 4 °C followed by centrifuging. The supernatant was discarded. The stained cell pellets were resuspended in FWB, transferred to FACS-tubes and subjected for analysis by flowcytometer.

2.2.3.1.2 Fixation and Permeabilization of Cells for Intracellular Cytokines

Fixation: Resuspended cells from previous steps were fixed in 4% paraformaldehyde for 10-20 min at 4 °C. The supernatant was removed and the cells were resuspended in FWB and then kept in the plate at 4 °C. Fixed cells were washed two times with FWB before next steps.

Permeabilizing: Fixed cells were resuspended in 0.3% Saponin (vortex and mix) in FWB for 10 min. The cells were centrifuged and the supernatant was discarded.

Staining for Intracellular Cytokines: The fixed/permeabilized cells were resuspended in 0.3% Saponin before intracellular staining for Foxp3, IFN-γ, IL4 and IL17. The resuspended cells were incubated with 2 µl of cocktails containing a pre-determined optimal concentration of a fluorochrome-conjugated anti-cytokine antibodies or appropriate negative control at 4 °C for 30 min in the dark. The cells were washed two times with 0.1% Saponin in FWB, then spun down at 2000 rpm for 2 min. The supernatant was discarded and cells were resuspended in FACS buffer. Cells suspensions were transferred to the tubes for acquisition on flowcytometer. For performing multiple colors staining, fluorochrome-labeled antibodies were added simultaneously to cell suspension in each well followed by incubation and washing steps as described above. All steps were performed in the cold and the samples protected from light during working with fluorescent antibodies. For all cytokines, data acquisitions were performed for cells at the same day for subsequent analysis. To assess cell numbers, all cells were acquired and analyzed with BD CellQuest pro software (BD). As shown in Table 2.1, cocktails of antibodies were prepared in a final volume of 200 µl.

Table 2.1 Flow cytometry antibodies` panel

Basic T cells		
Add 160 µl FWB*	+	10 µl of anti-CD3 (APC) (0.2 mg/ml) 10 µl of anti-CD4 (FITC) (0.5 mg/ml) 10 µl of anti-CD8 (PerCP-Cy5.5) (0.2 mg/ml) 10 µl of anti-B220 (PE) (0.2 mg/ml)
CD4 Memory/ Activated		
Add 160 µl FWB	+	10 µl of anti-CD4 (PerCP-Cy5.5) (0.2 mg/ml) 8 µl of anti-CD44 (FITC) (0.5 mg/ml) 10 µl of anti-CD62L (PE) (0.2 mg/ml) 12 µl of anti-CD69 (APC) (0.2 mg/ml)
CD8 Memory/ Activated		
Add 160 µl FWB	+	10 µl of anti-CD8 (PerCP-Cy5.5) (0.2 mg/ml) 8 µl of anti-CD44 (FITC) (0.5 mg/ml) 10 µl of anti-CD62L (PE) (0.2 mg/ml) 12 µl of anti-CD69 (APC) (0.2 mg/ml)
B cell Activation		
Add 160 µl FWB	+	10 µl of anti-CD19 (PerCP-Cy5.5) (0.2 mg/ml) 10 µl of anti-CD69 (APC) (0.2 mg/ml) 8 µl of anti-CD86 (FITC) (0.2 mg/ml) 14 µl of anti-MHC Class II (PE) (0.1 mg/ml)
Dendritic cells		
Add 170 µl FWB	+	10 µl of anti-CD11c (APC) (0.2 mg/ml) 10 µl of anti-PDCA-1 (PE) (0.2 mg/ml) 10 µl of anti-CD86 (FITC) (0.5 mg/ml)
Regulatory T cells		
Add 190 µl FWB	+	6/1 a: 10 µl anti-CD4 (PerCP-Cy5.5) (0.2 mg/ml) 6/2 a: 10 µl anti-CD8 (APC) (0.2 mg/ml) 6/2 b:10 µl anti-FoxP3** (PE) (0.2 mg/ml)
T helper 1/ Th2/ Th17		
Add 190 µl FWB	+	7a: 10 µl anti-CD4 (PerCP-Cy5.5) (0.2 mg/ml) 7b/1: 10 µl anti-IL17** (PE) (0.2 mg/ml) 7b/2: 10 µl anti-IFN-γ ** (FITC) (0.5 mg/ml) 7b/3: 10 µl anti-IL4** (APC) (0.2 mg/ml)

*FWB, FACS wash buffer

**These antibodies target antigens that are intracellular (IC), and thus require special treatment.

DCs, Dendritic cells*;* Tregs*,* Regulatory T cells*;* Th1/Th2/ Th17, T helper 1, 2 and 17cells; IL, interleukin.

2.2.3.2 Cells Subpopulation Determination

2.2.3.2.1 B cell and T cell Subsets

To examine cell population and subsets, the spleen cell suspensions were stained for basic T cell markers (CD3, CD4, CD8, B220 antibodies) and for B cell activation markers (CD19, CD69, CD86, MHC Class II antibodies) as shown in Table 2.1.

To determine the ratio of $CD4^+$ to $CD8^+$ T cells, single cell suspensions from spleens of G4 mice ($1x10^6$ cells) were incubated with Fc-block at room temperature and then stained for 15 min with CD4 (FITC-anti-mouse) and CD8 (PerCP-Cy5.5-anti-mouse antibodies). After two times washing with FACS buffer, cell subpopulations were determined. The percentage of $CD4^+$ cells was divided by the percentage of $CD8^+$ cells to calculate the CD4/CD8 ratio.

2.2.3.2.2 Dendritic cells Subsets

Dendritic cells (DCs) are specialized antigen-presenting cell (APCs), which control T-cell responses as well as the differentiation of T helper subtypes (Bailey et al., 2007; MacDonald et al., 2002; Pulendran et al., 1999). In addition, it has been reported that DCs can differentiate into $Foxp3^+$ Tregs in the presence of transforming growth factor-β (TGF-β), which could suppress autoimmune rejection of pancreatic islets in diabetic NOD mice (Luo et al., 2007). Therefore, the anti-CD11c, anti-plasmacytoid dendritic cell antigen 1 (PDCA-1) and anti-CD86, were used to identify DCs in G4 mice by flow cytometry.

2.2.3.2.3 Regulatory Tcells Subpopulation

Regulatory T cells (Tregs), primarily known as suppressor T cells, are a subpopulation of T cells which modulate the immune system, maintain tolerance to self-antigens and shift B cell to IgG4-producing plasmacytes. Tregs are involved in the development of various autoimmune diseases as well. Mouse models have proposed that modulation of Tregs can treat autoimmune disease. Regulatory T cells actively suppress activation of the immune system and prevent pathological self-reactivity, i.e. autoimmune disease. The severe autoimmune syndrome that results from a genetic deficiency in regulatory T cells evidences the critical role of Treg cells within the immune responses (Lu & Rudensky, 2009; Sakaguchi et al., 2009; Wildin et al., 2002; Wing & Sakaguchi, 2013). Recent research has shown that regulatory T-cells are determined by expression of the forkhead family transcription factor, (FOXP3,

forkhead box P3), which can be used as a good marker for mouse $CD4^+CD25^+$ T cells (Koyabu et al., 2013). Thus, to investigate the role of the Tregs in the pathophysiology of AIP, we analyzed the Tregs in the spleen of G4 mice using anti-CD4, anti-CD8 and anti-FoxP3 markers (Table 2.1).

2.2.3.2.4 T helper Subtyps

T helper subtypes have been implicated in the regulation of many immune responses (Romagnani, 1992). Several distinct cytokine secretion patterns have been defined among murine $CD4^+$ T cells. The different cytokine patterns lead to different functions of the types of T cell. T helper 1 (Th1) cells produce interleukin 2 (IL2), tumour necrosis factor-beta and gamma-interferon (IFN-γ), which is their effector cytokine. T helper 2 (Th2) cells are excellent helpers for B-cell antibody secretion, particularly IgE responses. Th2 cells are triggered by IL4 and express IL4, IL5, IL6 and IL10. Their effector cytokines are IL4 and IL5. T helper17 (Th17) cells are considered as a new subset of cells critical to the development of rheumatoid arthritis (RA). IL17 plays an important role in the pathogenesis of RA and the level of Th17 cells is associated with disease activity in RA (Kim et al., 2013).

Several researches suggested that the imbalance in T helper-subset activities might play a role in the pathogenesis of autoimmune diseases (Sudzius et al., 2013; Ulivieri & Baldari, 2013). Therefore, in this study intracellular cytokines (IFN-γ, IL4 and IL17) and T helper subsets of spleen lymphocytes were examined using flow cytometry in this study.

2.2.4 Statistical Analysis

All acquired data, measurements and information were exported to an Excel file. Genotype/phenotype correlation was analyzed for statistical significant with the R version of Happy in version 2.3 beta on Debian Linux. The data from all chromosomes were analysed together by an additive model. The significance of P values was confirmed for every SNP by comparison to 1000 permutations ($P<0.001$). The flanking regions of QTLs were determined manually by a drop of 1.5 to both sides to the peak. Scientific data and schemes were created and visualized using Power Point.

3 Results

3.1 Phenotyping Analysis of 4-way Autoimmune-prone Intercross Mouse Line

In this work, a genetically diverse mouse line was generated by intercrossing AIP-susceptible MRL/MpJ mice (Kanno et al., 1992; Sorg et al., 2008) and three resistant mice strains BXD2, NZM and Cast and their offsprings for several generations. The genetic diversity of four-way autoimmune-prone intercross mouse line is identified by the different morphological traits, such as weight, tail length or coat colour.

Phenotypic characterisation of spontaneous AIP in parental strain and in G4 mice was carried out at age of 6 month by scoring the severity of AIP-typical pancreatic lesions in H&E paraffin sections of pancreatic tissue specimens. Out of 351 G4 mice analysed, 12.8% of all animals (45 mice) displayed signs of parenchymal destructions and were classified as AIP stage 2 or 3 (Table 3.1). This number corresponds to 17.6% of the females (n=33) but only 7.4% of the males (n=12). Within 6 month-old G4 mice, stage 4 was not observed. The details are given in Table 3.1. Interestingly, similar percentages of G4 mice developed spontaneous arthritis (Ranea et al., 2013) and lupus nephritis (unpublished data from Susan Müller & Saleh Ibrahim).

Table 3.1 Summary of phenotyping: pancreatic histopathology of the fourth generation of four-way autoimmune-prone AIL

		AIP stage				Stage
Gender	Number	0	1	2	3	2+3(%)
Male	163	83	68	11	1	7.4
Female	188	53	102	32	1	17.6
Both	351	136	170	43	2	12.8

Pancreatic sections of 351 G4 mice (6 months old) were stained with Haematoxylin & Eosin, and evaluated applying a scoring system from 0 to 4 as further described in the methods section (2.2.1.2.1). AIP, autoimmune pancreatitis.

As previously described, histological analyses of H&E stained sections of pancreata from the fourth generation (G4) AIL mice clearly showed that these mice developed pancreatic lesions at the age of 6 months (Figure 3.1).

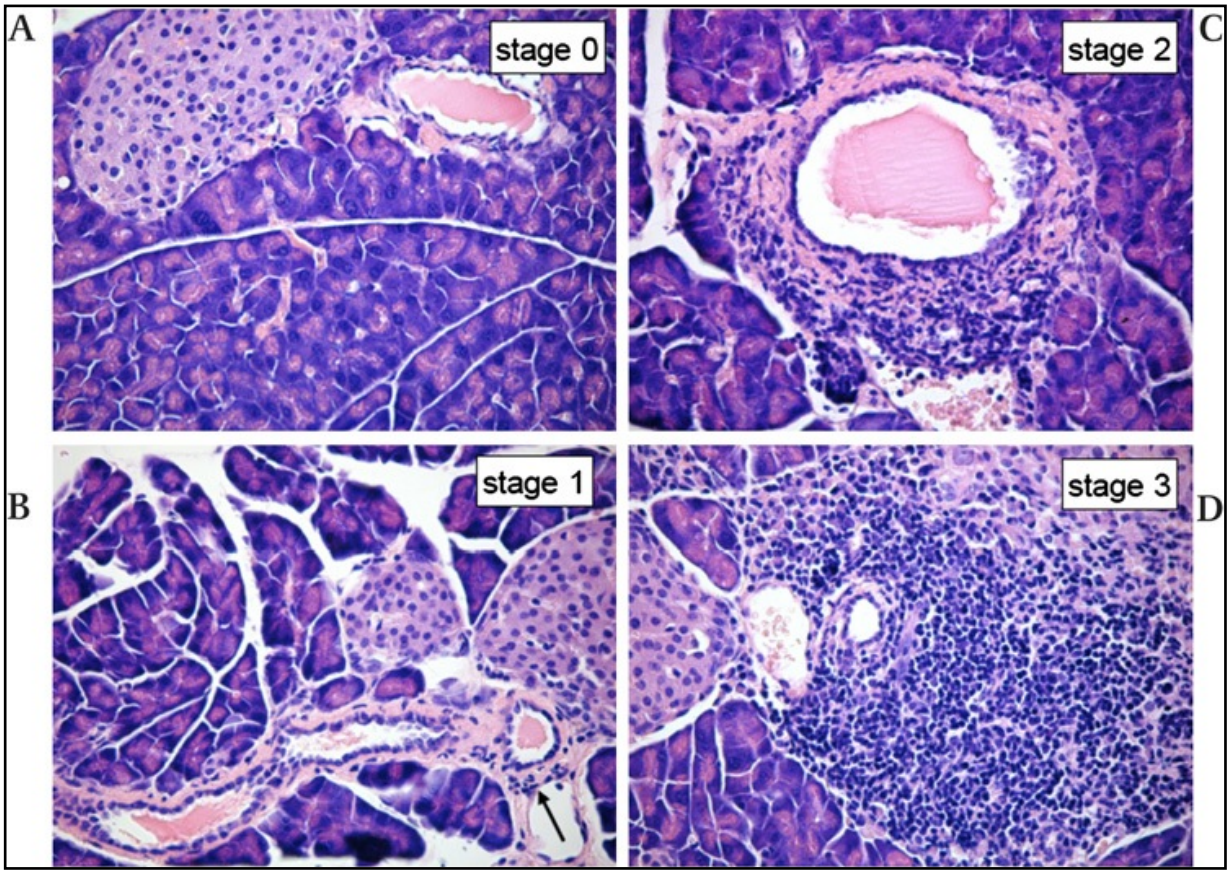

Figure 3.1 Representative Haematoxylin & Eosin–stained histological sections of the pancreas in G4 Mice.

Pancreatic sections of G4 mice were stained with Haematoxylin & Eosin (original magnification X-200). Histopathological findings of pancreatic lesions in G4 mice are ranging from stage 0 (A) to 3 (D). (A) Healthy pancreas; stage 0. (B) Preserved exocrine and endocrine pancreas. One larger duct shows a minimal lymphocytic infiltration of the sub-epithelial layer (arrow); stage 1. (C) Moderate periductal lymphocytic infiltration; beginning destruction of acinar tissue; stage 2. (D) Severe periductal inflammation with moderate parenchymal destruction; stage 3.

In accordance with previous studies (Fitzner et al., 2009; Qu et al., 2002; Sorg et al., 2008), immunohistochemistry studies revealed that $CD4^+$ and $CD8^+$ T lymphocytes were predominantly found in pancreatic inflammatory lesions. Furthermore, CD45R ($B220^+$) B-lymphocytes and few F4/80-expressing cells (macrophages) were observed in pancreas (Figure 3.2). While these findings are comparable to histopathological changes in human AIP, fibrosis was minimal and only restricted to stage 3 of the disease.

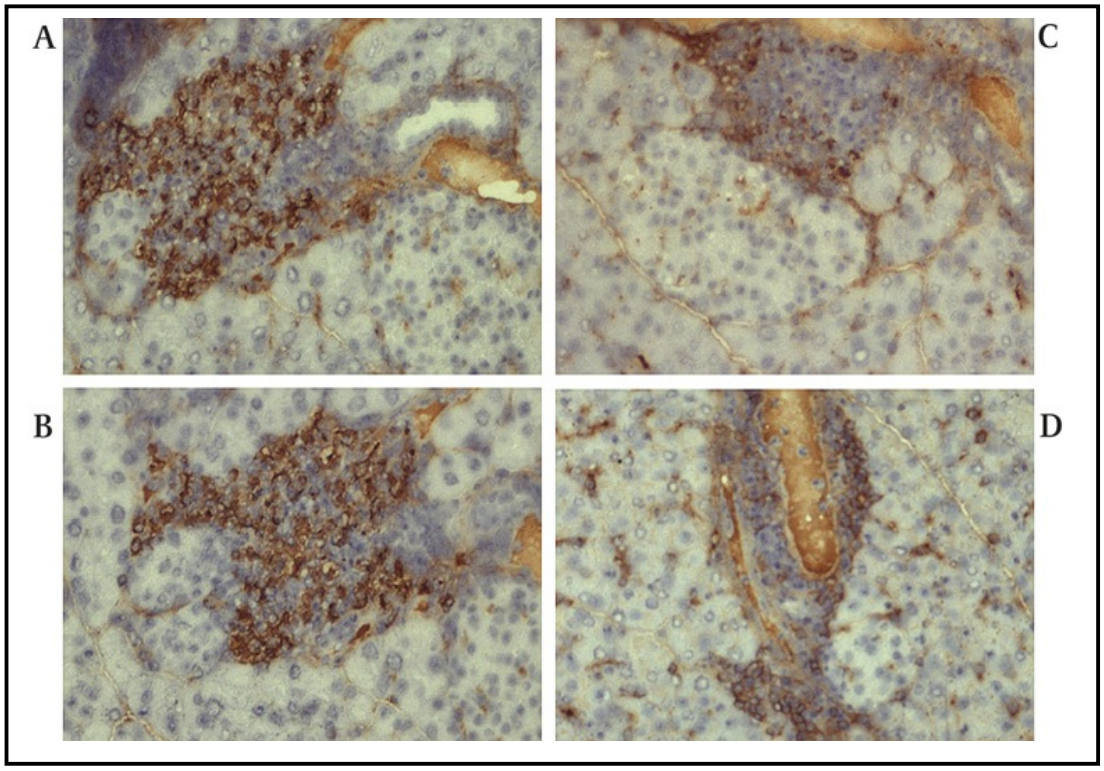

Figure 3.2 Immunohistochemical analysis of leukocytes infiltration within the mouse pancreas (autoimmune pancreatitis stage 2).

Tissue sections were stained with the ABC staining kit, using primary antibodies against (A) CD4, (B) CD8 (T lymphocytes), (C) F4/80 (macrophages), and (D) CD45R (B220; B cells). Note the pronounced cellular infiltration within the focal inflammatory lesions (original magnification x100).

Since the inflammatory lesions in kidney, lymph nodes and spleen of MRL mice have been broadly reported (Jabs et al., 2001; Matsumoto et al., 2003; Uchida et al., 2002; J.-Q. Yang et al., 2004), the tissues of these organs were preserved for further histopathological examinations to evaluate the general signs of health and the possibilities of other diseases associated with AIP.

3.2 Identification of Quantitative Trait Loci for Spontaneous AIP

The genetic control of susceptibility to AIP was analyzed by an association study performed with the software package R-HAPPY. It determines the statistical significance of genetic loci based on ANOVA tests (Table 3.2). Empirical significance levels were assessed by 1000 permutations. A cut-off of 0.001 in the empirical P value, equivalent to one false positive per 1,000 associated SNPs, was defined and those SNPs above this threshold were considered significant. Empirical P values < 0.01 were considered suggestive. Plots of whole genome linkage map for autoimmune pancreatitis are represented in Figure 3.3 and Figure 3.4

that include details on chromosomes 2, 4, 5 and 6. Five QTLs were identified with logarithmic P values > 3, which were linked to the severity of AIP. Correspondingly, the first QTL was mapped on chromosome 2 (peak in rs6222797; ~32.94 Mbp CI). The next two QTLs were mapped to chromosome 4 (one peak in rs3676928; ~ 41.31 Mbp CI and the other peak in rs6287606; ~9.38 Mbp CI), another one was located on chromosome 5 (peak in rs3667334; ~ 42.74Mbp CI) and finally one was mapped to chromosome 6 (peak in rs3695724; ~ 60.30 Mbp CI) (Table 3.2, Figure 3.3 & Figure 3.4). Logarithmic P values of these five QTLs (subsequently termed *AIP1-5*) varied from 3.2 to 5.4. The mean confidence interval of each QTL was approximately 12 cM. Although a clear sex effect on the AIP pathological phenotype was observed (Table 3.1), analysing the genotyping data with sex as a co-variant showed no sex-effect or X-linked QTLs in these mice. Similar observation in the model of collagen-induced arthritis (Bauer et al., 2004) revealed that the effect of X-linked genes may be too small to be verified using this approach.

Table 3.2 Summary of QTLs controlling AIP

QTL	Chr.	Peaks (Mbp) [a]	CI (Mbp)	- log P [b]	Overlapping autoimmunity QTL [c]
AIP1	2	64.9, 74.7	56.3 to 81.9	4.4	*EAE21*
AIP2	4	18	7.1 to 32.3	3.2	*Aaom1*, *Lxw1*
AIP3	4	82.4, 103.6	81.3 to 120.7	3.2	*Sle2*, *Adaz1*, *Agnm2*, *Pgia27*, *Psds1*
AIP4	5	83.5	74.8 to 96.6	3.5	*Cdcs10*
AIP5	6	121.2	111.2 to 133.9	5.4	*Cia3*

The table presents all QTLs found significant by 1000 permutations, their chromosomal location, as well as their overlap with known autoimmunity QTL. The log P value presents the likelihood of observing the effect by chance as indicated by a single run of HAPPY.

AIP, autoimmune pancreatitis; QTL, quantitative trait loci; CI, confidential interval; MbP, mega base pair; Chr., chromosome.

a. Position according to the NCBI Build 37

b. -log P value of the association.

c. QTLs retrieved from MGI database.
(http://www.informatics.jax.org/searches/allele_form.shtml)

The highest logarithmic P values were observed for two QTLs controlling AIP on chromosomes 2 and 6 (Table 3.2). The details of these QTLs are highlighted below (Figure 3.3).

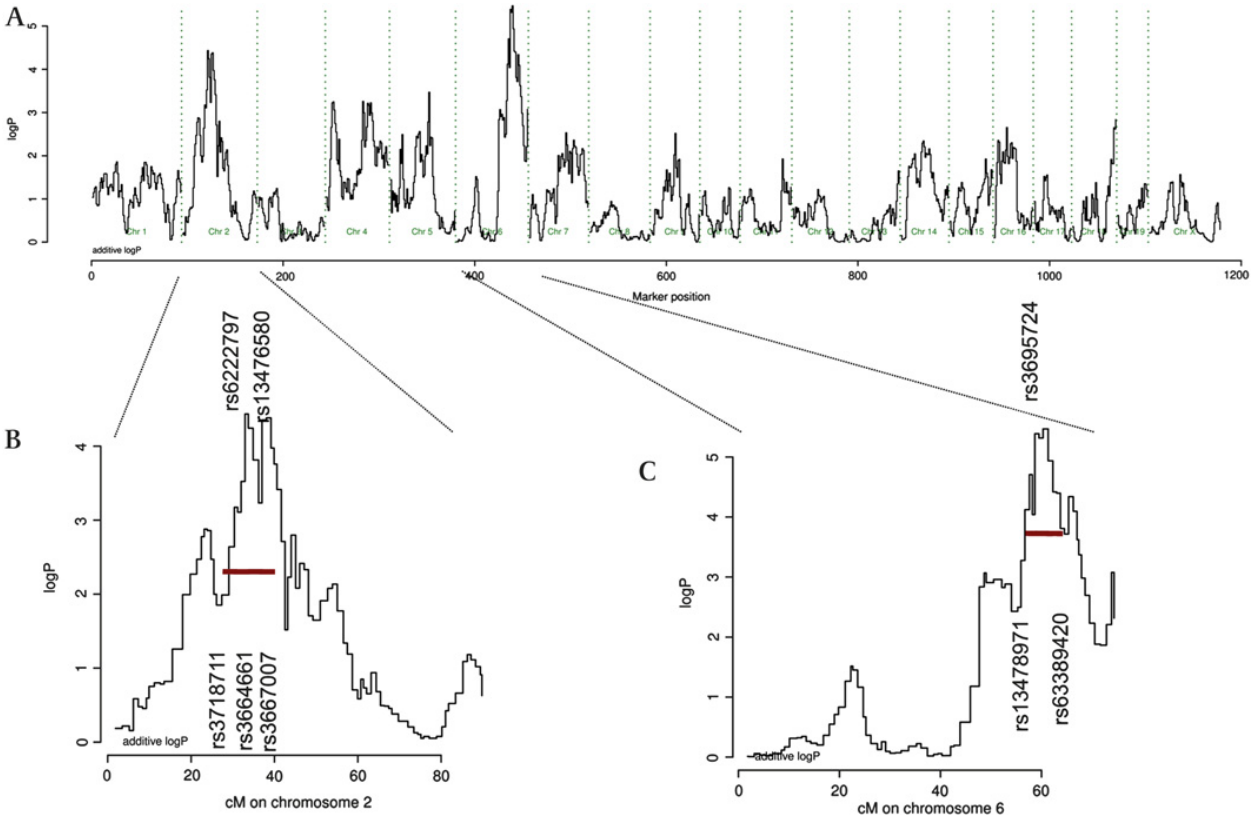

Figure 3.3 Whole genome linkage map for spontaneous autoimmune pancreatitis I (detailed on chromosomes 2 and 6).

Plots of autoimmune pancreatitis QTLs, on full genome (A), chromosome 2 (B), and chromosome 6 (C). Log-likelihood values were determined using HAPPY. Logarithmic P values are presented on the y-axis in relation to positions of the marker loci (in megabases) along the chromosome, which are on the x-axis. Confidence intervals and flanking markers of the QTLs are indicated.

3.3 Selected Candidate Genes

Although a fine mapping of the QTLs still has to be accomplished, a first search for possible candidate genes was performed. Therefore, we focused on the two QTLs with logarithmic P values higher than 4, which are located on chromosomes 2 and 6 (Table 3.3). The most promising findings in these two chromosomes, including their position and SNPs found next to the peaks of the QTLs are summarised in Table 3.3. Moreover, details of SNPs

found next to the peaks of AIP QTLs (*AIP1* and *AIP5* QTLs) are given in Table 3.4. Subsequently, these potential candidate genes are discussed in detail in the next chapter.

Table 3.3 Selected candidate genes of AIP QTLs in chromosomes 2 and 6

QTL	Chr.	Gene[a]	Description	Position (Mbp)[b]	SNP next to peak of QTL	Distance to peak of QTL (Mbp)
AIP1	2	*Hnrnpa3*	Heterogeneous nuclear ribonucleoprotein A3	75.5	rs13476580	0.8
AIP1	2	*Nfe2l2*	Nuclear factor, erythroid derived 2, like 2	75.5	rs13476581	0.8
AIP1	2	*Ssb*	Sjogren syndrome antigen B	69.7	rs6222797	4.8
AIP1	2	*Ubr3*	Ubiquitin protein ligase E3 component n-recognin 3	69.7	rs6222798	4.8
AIP5	6	*Clec4a2*	C-type lectin domain family 4,member a2	123.1	rs3695724	1.9
AIP5	6	*Pparg*	peroxisome proliferator activated receptor gamma	115.4	rs3695724	5.8

The table shows the candidate genes of the *AIP 1* and *AIP5* QTLs (with the highest logarithmic P values varied from 4.4 to 5.4) as well as their chromosomal location and position.

AIP, Autoimmune pancreatitis; QTL, quantitative trait loci; Chr., chromosome; Mbp, mega base pair; SNP, single nucleotide polymorphism.

a. Genes retrieved from MGI database.
(http://www.informatics.jax.org/searches/allele_form.shtml)

b. Position according to the NCBI Build 37

Table 3.4 SNPs next to the peaks of AIP QTLs on chromosomes 2 and 6

QTL	Chr.	SNP [a]	Position (cM).[b]	Position (bp) [c]	- log P [d]
AIP1	2	rs6222797	32.94	65119051	4.44
AIP1	2	rs13476553	33.72	66967901	4.25
AIP1	2	CEL.2_73370728 *	37.01	73314518	4.36
AIP1	2	rs13476580	38.05	74881866	4.38
AIP5	6	rs13478977	56.71	112524461	4.12
AIP5	6	rs6393943	57.60	114407245	4.71
AIP5	6	rs6401637	58.19	116090323	4.04
AIP5	6	mCV23042866**	58.68	116756729	5.39
AIP5	6	rs13478997	59.49	118072426	5.32
AIP5	6	rs3695724	60.30	120007151	5.47
AIP5	6	rs13479006	61.31	121299359	4.94
AIP5	6	gnf06.122.747*	62.25	124006411	4.42
AIP5	6	rs13479014	63.00	125048587	4.40
AIP5	6	CEL.6_130920075*	65.36	130188076	4.34
AIP5	6	rs3722480	66.29	131308308	4.10

AIP, Autoimmune pancreatitis; QTL, quantitative trait loci; Chr., chromosome; SNP, single nucleotide polymorphism; bp, base pair; cM, centimorgan.

a. SNPs symbols and description retrieved from MGI database.
(http://www.informatics.jax.org/searches/allele_form.shtml)

b. Position retrieved from Ensembl.
(http://www.ensembl.org/Mus_musculus/Location)

c. Position according to the NCBI Build 37
(http://www.ncbi.nlm.nih.gov/snp/?term).

d. -log P value of the association.

* Position according to (http://www.well.ox.ac.uk/flint-old/distrib/chr6.build37.map)

** Identical marker as rs30223567

The next three AIP QTLs were mapped on chromosomes 4 (n=2) and 5 (with logarithmic P values of 3.2 ≥ 3.5) (Table 3.2 and Figure 3.4). The details of these QTLs and other overlapping autoimmunity QTLs are summarized in the Table 3.5 and Table 3.6.

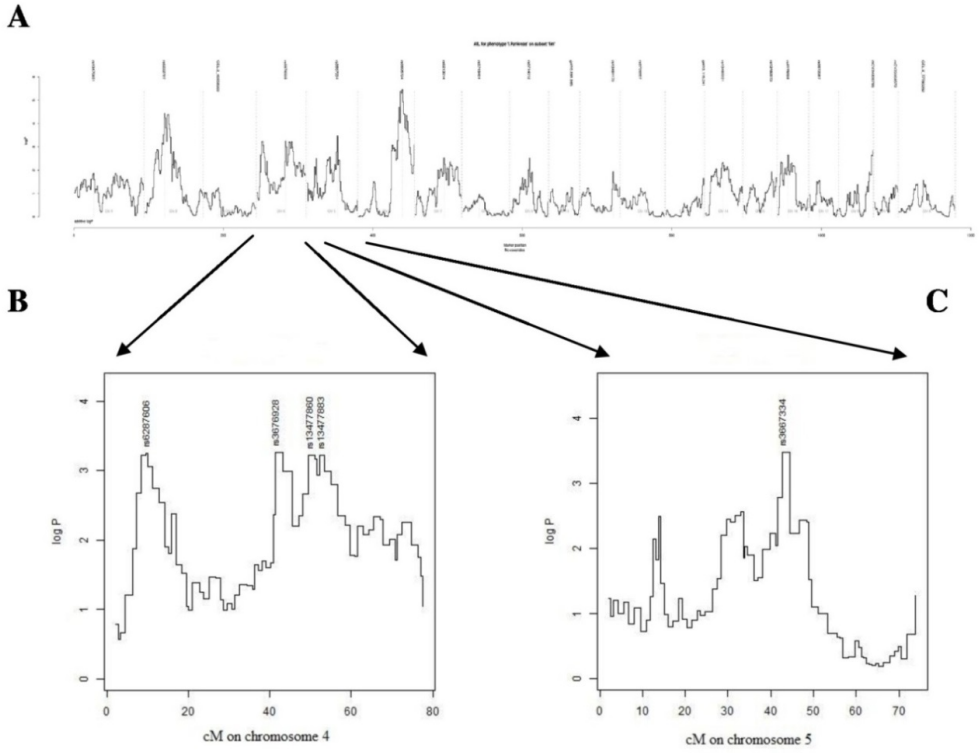

Figure 3.4 Whole genome linkage map for spontaneous autoimmune pancreatitis II (detailed on chromosomes 4 and 5).

Plots represent autoimmune pancreatitis QTLs on full genome (A), chromosome 4 (B), and chromosome 5 (C). Log-likelihood values were determined using HAPPY. Negative logarithmic P values are presented in relation to positions of the marker loci along the chromosome (cM, centimorgan). The x-axis represents position in Mbp and the y-axis shows -log P value of the association.

Table 3.5 SNPs next to the peaks of AIP QTLs on chromosomes 4 and 5

QTL	Chr.	SNP [a]	Position (cM)[b]	Position (bp)[c]	-log P [d]
AIP2	4	mCV22939387	8.34	15394344	3.22
AIP2	4	rs6287606	9.39	18118213	3.25
AIP3	4	rs3676928	41.31	82765182	3.26
AIP3	4	rs13477860	49.53	97344236	3.21
AIP3	4	rs13477883	52.32	103962063	3.23
AIP4	5	rs3667334	42.75	83042043	3.47

Chr.; chromosom, SNP; single nucleotide polymorphism, cM; centi morgan, bp; base pair

a. SNPs symbols and description retrieved from MGI database. (http://www.informatics.jax.org/searches/allele_form.shtml)

b. Position retrieved from Ensembl. (http://www.ensembl.org/Mus_musculus/Location)

c. Position according to the NCBI Build 37 (http://www.ncbi.nlm.nih.gov/snp)

d. -log P value of the association.

3.4 AIP QTLs Overlap with QTLs for Other Autoimmune Diseases

Data from Table 3.2 demonstrates that some of the newly identified AIP QTLs overlapped with known arthritis and lupus QTLs, e.g. *AIP1* with *EAE21* on chromosome 2, *AIP3* with *Sle2* and *Psds1* on chromosome 4, and *AIP5* with *Cia3* on chromosome 6 (McIndoe et al., 1999; Santiago-Raber et al., 2007). The details of these QTLs are given in Table 3.6.

Table 3.6 Overlapping newly indentified AIP QTLs in G4 mice with autoimmunity QTLs

QTL	Chr.	Overlapping QTL[a]	Description	Position (Mbp)[b]	SNP next to peak of QTL[c]	Distance to peak of QTL (Mbp)
AIP2	4	*Aaom1*	Autoimmune aoritis in MRL mice 1	24.3-46.3	mCV22939387	6.3
AIP2	4	*Lxw1*	Lupus BXSB x NZW 1	25.6	rs6287606	7.5
AIP3	4	*Sle2*	Systemic lupus erythmatosus susceptibility 2	82.8	rs3676928	0.1
AIP3	4	*Adaz1*	Anti-dsDNA antibody production in NZM 1	100.1	rs13477860	2.76
AIP3	4	*Agnm2*	Autoimmune glomerulonephritis in MRL 2	118.4	rs13477883	14.4
AIP3	4	*Pgia27*	Proteoglycan induced arthritis 27	87.1	rs3676928	4.7
AIP3	4	*Psds1*	Psoriasis-like skin disease severity 1	76.66	rs3676928	6.1
AIP4	5	*Cdcs10*	Cytokine deficiency colitis susceptibility 10	93.06	rs3667334	9.8

The table provides details of overlapping autoimmunity QTLs with AIP QTLs (termed as *AIP 2, 3, 4* with the highest -log P value of 3.2 to 3.5), their chromosomal location and position.

AIP, Autoimmune pancreatitis; QTL, quantitative trait loci; SNP, single nucleotide polymorphism; Mbp, mega base pair.

a. QTLs symbols and description retrieved from MGI database. (http://www.informatics.jax.org/searches/allele_form.shtml)

b. Position retrieved from Ensembl (http://www.ensembl.org/Mus_musculus/Location)

c. Position according to the NCBI Build 37

3.5 Detection of Immune Response Phenotypes

To identify immune cells associated with AIP, flow cytometry-based phenotypic analysis was performed on spleenocytes of G4 mice (n=351) and parental strains (n=54). The percentages of T cells and their subpopulations, B cells and DCs were determined using specific surface markers. Tregs and T helper subtypes were determined by immunostaining for intracellular markers. The mean values of the percentages of subpopulations are summarised in Table 3.7.

Table 3.7 Summary of immune cell phenotypes of mice splenocytes detected by flow cytometry

Subpopulation	Mean ± SEM*		Description
	G4 Mice	Parental Strains	
$CD3^+ CD4^+$	15.68 ± 5.45	21.16 ± 6.918	T helper cells
$CD3^+ CD4^-$	9.97 ± 4.15	13.17 ± 9.327	T cells
$CD3^+ CD8^+$	8.48 ± 4.05	11.14 ± 8.897	T suppressor/cytotoxic lymphocyte
$CD3^+ CD8^-$	17.18 ± 5.61	23.19 ± 7.146	T cells
$CD4^+ CD8^+$ (DP)	0.57 ± 0.36	0.44 ± 0.711	Doubel positive T cells
$CD4^- CD8^-$ (DN)	35.01±16.32	47.29 ± 8.772	Double negative leukocytes
$CD8^+ B220^+$	19.07 ± 6.40	0.60 ± 0.828	T cells/ T cytotoxic/suppressor
$CD4^+ B220^+$	0.80 ± 0.45	1.13 ± 1.561	T cells/ T helper
$CD44^+ CD69^+$	0.40 ± 0.65	0.79 ± 0.513	Memory T cells/effector Tcells
$CD44^+ CD62L^+$	16.21±10.12	12.48 ± 5.127	Naive/memory/effector T cells
$CD44^+ CD8^+$	4.76 ± 3.34	4.84 ± 2.366	Memory CD8 T cells/age
$CD8^+ CD62L^+$	6.18 ± 3.79	9.69 ± 7.878	Memory CD8 T cells/central in pb
$CD8^+ CD69^+$	0.37 ± 0.35	0.67 ± 0.790	Activated CD8 cells
$CD44^+ CD4^+$	12.97 ± 4.48	15.40 ± 7.153	Memory CD4 T cells
$CD4^+ CD69^+$	0.31 ± 0.59	0.60 ± 0.319	Tregs
$CD62L^+ CD4^+$	7.72 ± 4.66	9.30 ± 6.884	T helper / Th1/Th2 differentiation
$CD19^+ CD69^+$	0.28 ± 0.19	0.16 ± 0.117	B cells, Activated B cells (B^{act})
$CD19^+ CD69^-$	37.34 ± 11.26	16.35 ± 6.958	B cells
$CD19^+ CD86^+$	0.43 ± 0.69	0.39 ± 0.343	B cells, B^{act}
$CD19^+$ MHC $classII^+$	33.02 ± 11.53	11.85 ± 4.804	B cell
$CD69^+$ MHC $classII^+$	0.27 ± 0.20	0.21 ± 0.126	APC,Dcs and B cells
$CD86^+$ MHC $classII^+$	0.46 ± 0.82	0.64 ± 0.628	APC
$CD11c^+ PDCA1^+$	0.74 ± 0.46	0.49 ± 0.377	Dendritic/plasmacytoid dendritic cells
$CD11c^+ CD86^+$	0.33 ± 0.35	0.16 ± 0.170	Dcs
$CD11c^+ PDCA1^-$	1.90 ± 1.00	2.02 ± 1.285	Dcs
$CD11c^- PDCA1^+$	2.63 ± 3.70	2.78 ± 5.774	Plasmacytoid dendritic cells (PDcs)
$CD86^+ PDCA1^+$	0.48 ± 0.62	0.22 ± 0.288	PDcs
$CD4^+ FoxP3^+$	0.45 ± 0.42	0.06±0.038	Tregs (helper)
$CD8^+ FoxP3^+$	0.14 ± 0.14	0.04±0.036	Tregs(cytotoxic or suppressor)
$CD4^+ IL17^+$	0.31 ± 0.29	0.02 ± 0.023	T helper 17
$CD4^+$ IFN-γ^+	0.51 ± 0.63	0.04 ± 0.032	T helper 1
$CD4^+ IL4^+$	0.21 ± 0.20	0.31 ± 0.182	T helper 2

*Results are expressed as mean ± SEM of total splenocytes (×106) of G4 four-way AIL mice (n=351) and parental strain (MRL,BXD, NZM,CAST mice)(n=54)

CD; Cluster of differentiation, DP; Double positive, DN;double negative, FoxP3;forkhead-box-protein P3, PDCA-1; plasmacytoid dendritic cell antigen-1, APC; Antigen-presenting cells, MHC classII; major histocompatibility complex class II, Tregs; Regulatory T cells, IL17;interleukin 17, IFN-γ; interferon gamma, IL4; interleukin 4, Dcs; dendritic cells.

The ratio of $CD3^+CD4^+$ T cells to $CD3^+CD8^+$ T cells (CD4/CD8 ratio) was also calculated. The CD4/CD8 ratios of splenic T lymphocytes of 6-month-old mice were 1.849 in G4 mice and 1.899 in parental strains (Table 3.7).

We found a significant positive correlation between autoimmune pancreatitis and T helper cells ($CD4^+$), activated T helper ($CD4^+ CD44^+$) in 6 months G4 old mice (significant with Benjamini Hochberg FDR <0.05) (Table 3.8). Additionally, a negative correlation was observed between AIP and $CD8^+ CD44^-$ phenotype.

Table 3.8 Correlation between AIP and splenic B and T Lymphocytes

phenotype	correlation coefficient*	P-value	corrected P-value**
$CD4^+ CD44^+$	0.19	0.0003	0.0153
$CD4^+ CD69^+$	0.16	0.0030	0.0436
$CD8^+CD44^-$	-0.16	0.0023	0.0436

* Spearman`s rho

** Benjamini Hochberg FDR<0.05

These findings are in line with numerous studies showing that T cells play role in development of acute and chronic inflammatory diseases. In particular, $CD3^+CD4^-CD8^-$ (double negative) T cells and their potential pathogenic role in inflammation and autoimmunity (D'Acquisto & Crompton, 2011) might be of interest.

An early development of pancreatitis with abundant infiltration of activated Foxp3-positive cells was observed in MRL/Mp mice treated with poly I:C (Koyabu et al., 2013), suggesting that Tregs may be involved in the development of pancreatitis in these mice. We asked whether QTL controlling activated and/ or Treg cells overlapped with AIP QTLs. Interestingly, screening for phenotype/genotype linkage in chromosome 2 displayed overlapping for all of 12 SNPs that were significant for *AIP1* QTL. High logarithmic P values ($3.2 \geq 7.11$) were observed for linkage between these SNPs and the following three phenotypes: $CD4^+ FOXP3^+$, $CD8^+ FOXP3^+$, $CD8^+ CD69^+$ (Figure 3.5). The table below illustrates the details of these SNPs (Table 3.9).

Table 3.9 SNPs on Chr 2 controlling AIP located in QTL of the three phenotypes (Tregs and activated Tcells)

Marker [a]	Position (cM) [b]	$CD4^+FOXP3^+$ -log P [c]	$CD8^+FOXP3^+$ -log P [c]	$CD8^+CD69^+$ -log P [c]
rs13476530	30.374	7.11	6.47	4.55
rs4223189	31.170	6.39	5.59	4.42
CEL.2_63143553	32.000	5.62	4.57	4.90
rs6222797	32.941	5.99	4.99	6.46
rs13476553	33.723	6.77	5.52	6.37
rs6381994	34.881	6.75	5.70	4.30
rs3664661	36.188	6.38	5.34	3.80
CEL.2_73370728	37.009	5.67	4.47	3.31
rs13476580	38.053	5.04	4.08	4.07
gnf02.076.311	39.169	4.90	4.07	5.27
CEL.2_79237503	39.910	5.79	5.79	5.69
rs3722345	40.600	4.95	4.94	6.02

The updated list of SNPs located on *AIP1* QTL showed overlapping for $CD4^+FOXP3^+$, $CD8^+FOXP3^+$ and $CD8^+CD69^+$ phenotypes on Chr2, as well as their position and -log P value.

a. Position according to the NCBI Build 37

b. Position retrieved from Ensembl. (http://www.ensembl.org/Mus_musculus/Location)

c. -log P value of the association.

The first SNP in Table 3.9 (rs13476530) with the highest logarithmic P value (-log P value=7.11), additionally overlaps with four previously identified QTLs: Collagen-induced arthritis QTL 2 (*Cia2*), Collagen-induced arthritis QTL 4 (*Cia4*), Experimental allergic encephalomyelitis susceptibility 21 (*Eae21*) and Experimental allergic encephalomyelitis susceptibility 33 (*Eae33*).

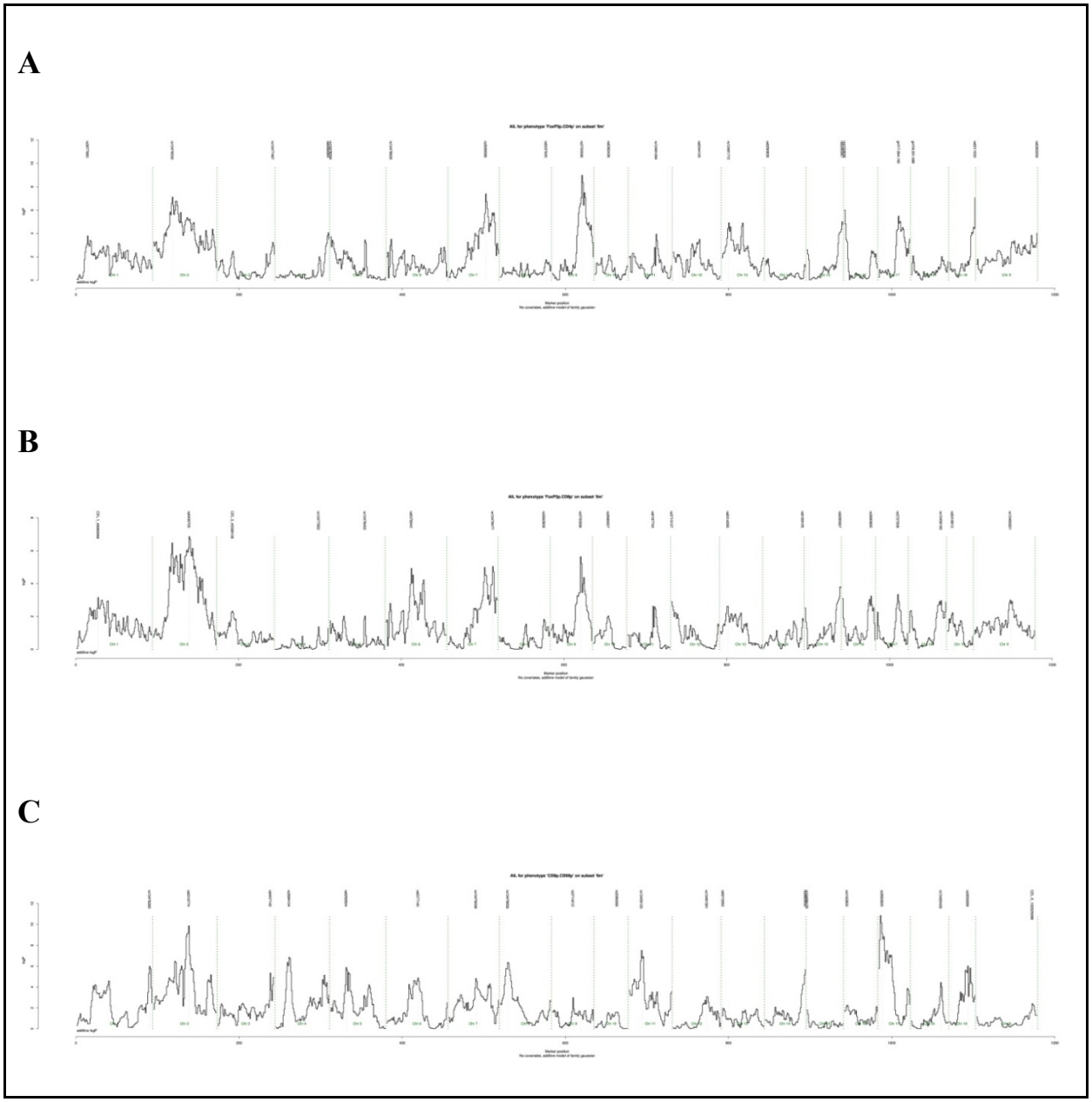

Figure 3.5 Whole-genome linkage map for AIP and Treg markers/activated T cells as covariates in G4

The figures show genotype/phenotype overlapping of QTLs controlling AIP with (A) $CD4^+FOXP3^+$, (B) $CD8^+FOXP3^+$ and (C) $CD8^+CD69^+$ phenotypes, which were used as covariates. Logarithmic p values are presented in relation to positions of the marker loci along the chromosome. The x-axis represents position in Mbp and the y-axis shows -log P value of the association.

In Figure 3.5/B a locus (rs6378343, 6:63823084bp, ~ 32.20cM, -log P value =4.9) overlaps with collagen-induced arthritis QTL 6 (*Cia6* on Chr6:4464884-87426034). This region contains four genes, which support a previous suggestion that $CD8^+$ T cell could play a protective role in some AID (Gravano & Hoyer, 2013; McKinney et al., 2010). These genes are CD8 antigen alpha chain (*Cd8a* on Chr6:71373427-71379171), CD8 antigen, beta chain 1

(*Cd8b1* on Chr6:71322812-71337451), interleukin 23 receptor (*Il23r* on Chr6:67422932-67491855) and interleukin 12 receptor beta 2 (*Il12rb2* on Chr6:67291318-67376188).

As it can be seen in Figure 3.5 A & B, two other peaks on Chr. 7 (SNP next to the peak: rs3699086, ~52.28cM, with -log P value = 7.38 and 4.98) and Chr. 9 (SNP next to the peak: rs3700596, ~43.49 cM, with -log P value = 8.97 and 5.63) linked to the $CD4^+$ $FOXP3^+$ and $CD8^+$ $FOXP3^+$ phenotypes as well. The most important findings in the region on Chr. 7 are summarized in Table 3.10.

Table 3.10 QTLs or genes located next to the peak SNP (rs3699086) on chromosome 7

Symbol [a]	type	Name	Position (bp) [b]
Il4ra	gene	interleukin 4 receptor, alpha	125552282-125579472
Il4ppq	QTL	IL4 producing potential	124914439-129217600
Lat	gene	linker for activation of T cells	126363827-126369534
Il18bp	gene	interleukin 18 binding protein	102015079-102018155
*Igsf6**	gene	immunoglobulin superfamily, member 6	121064067-121074530
Eae26	QTL	experimental allergic encephalomyelitis susceptibility 26	115565687-115565775
Cia7	QTL	Collagen-induced arthritis QTL 7	85716387-112934612
Idd27	QTL	insulin dependent diabetes susceptibility 27 QTL	79376386-119886157
Cd19	gene	CD19 antigen	126408450-126414870

QTL, Quantitative trait loci; bp, base pair.

a. SNPs symbols and description retrieved from MGI database. (http://www.informatics.jax.org/searches/allele_form.shtml)

b. Position retrieved from Ensembl. (http://www.ensembl.org/Mus_musculus/Location)

*This gene is mapped to the inflammatory bowel disease 1 locus (Bates et al., 2000).

There was a significant association between the locus (rs3700596:~ 43.49cM) on Chr.9 and $CD4^+$ $FoxP3^+$ (-log P value = 8.975), $CD8^+$ $FoxP3^+$ (-log P value = 5.63). Interestingly, the T lymphocyte fraction gene (*Tlf*)[8] maps also in this region (~ 52.24 cM), which shows association with autoimmune diabetes (Pearce, 1998; Pearce et al., 1995).

In the next step, the results of immunophenotyping are checked in the same way for linkage between AIP QTLs and Th1, Th2 andTh17 phenotypes. Based on the role of IFN-γ, a pro-inflammatory cytokine released by Th1 cell, in the pathogenesis of autoimmune pancreatitis, a phenotype/genotype association analysis was performed. Our result in Figure

[8] http://www.informatics.jax.org/marker/MGI:109546

3.6 shows the overlap between AIP QTL and the region controlling $CD4^+$ IFN-γ^+ phenotype (Th1 cells) for the two following SNPs (both of them are in *AIP2* QTL):

- rs6287606 on Chr. 4 at 9.388 cM (-log P value = 9.01). The previously mapped QTLs in this region are and *Lxw1* (lupus BXSB x NZW 1) and *Aaom1* (autoimmune Aortitis in MRL mice1).
- mCV22939387 on Chr.4 at 8.341 cM (-log P value = 5.13), which overlaps with *Nbwa2* (NZB and NZW autoimmunity 2 QTL) on Chr.4 at 34892515-34892635 bp. This locus contains the interferon kappa gene (*Ifnk)* on Chr. 4 at 35152056-35154005 bp.

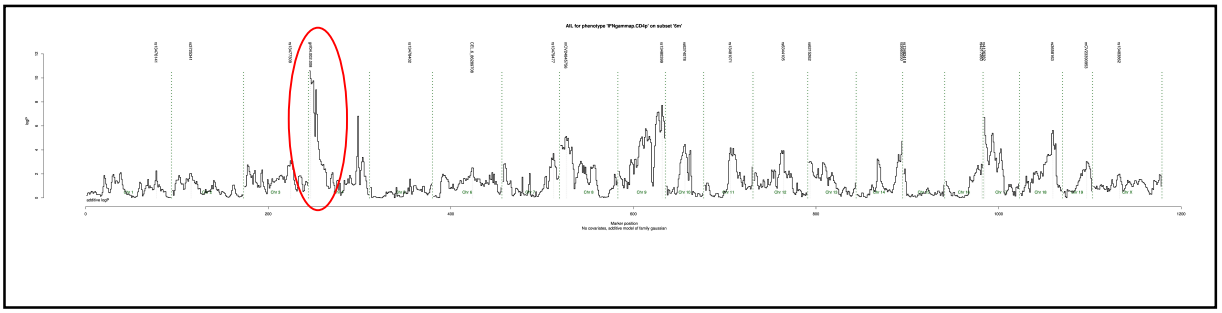

Figure 3.6 Whole-genome linkage map for AIP and $CD4^+$ IFN-γ^+ phenotype as covariant.
The plot shows relationship between AIP QTLs and Th1 marker as phenotype in G4 mice. The x-axis represents position in Mbp and the y-axis shows -log P value of the association.

As can be seen in Figure 3.7, the five SNPs (on Chr. 2) show overlapping for the region controlling $CD4^+$ $IL4^+$ (Th 2) phenotype:

- rs13476530 at position 30.37 cM (-log P value = 3.22)
- rs13476553 at position 33.72 cM (-log P value = 3.54)
- gnf02.076.311 at position 39.17 cM (-log P value =3.62)
- rs3722345 at position 40.60 cM (-log P value = 4.52)
- and CEL.2_79237503 at position 39.90 cM (-log P value = 4.54).

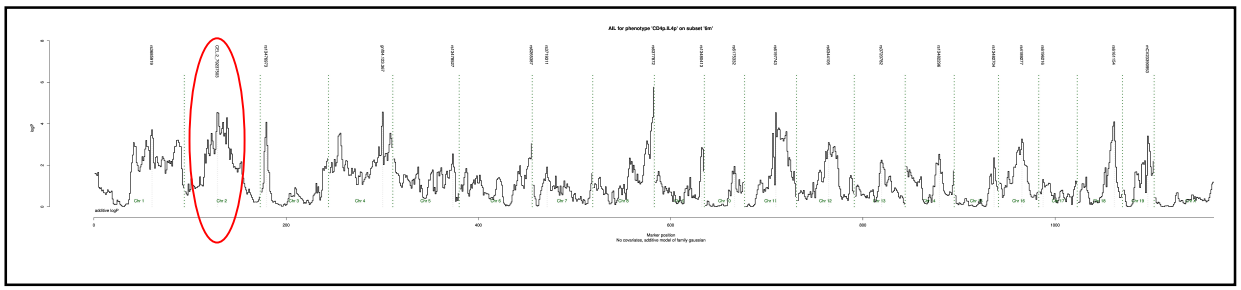

Figure 3.7 Whole-genome linkage map for AIP and $CD4^+$ $IL4^+$ as a covariate in G4 mice
Logarithmic P values are presented in relation to positions of the marker loci along the chromosome. The x-axis represents position in Mbp and the y-axis shows -log P value of the association.

The phenotype/genotype association and overlapping between AIP QTLs and several other immunologic QTLs was checked and provided relevant information about possible mechanisms underlying QTLs function. In *AIP5* QTL region, out of 16 SNPs with high logarithmic P values for AIP, five SNPs displayed overlapping for the region associated with $CD4^+$ $IL17^+$ phenotype, whereas in *AIP2* QTL region overlap was observed for 2 of 5 SNPs that linked with AIP (Figure 3.8 and Table 3.11). As can be seen in Figure 3.8, the same two SNPs controlling Th1 phenotype show overlapping for the region controlling $CD4^+$ $IL17^+$ (Th17) phenotype as well.

Table 3.11 SNPs on chromosomes 4 and 6 controlling AIP located in QTL and linked with $CD4^+IL17^+$ phenotype

SNPs [a]	Chr.	Position [b]		-log P	Overlapping autoimmune QTLs/Gene
		(cM)	(Mbp)		
mCV22939387	4	8.341675	15.39	4.609	*Nbwa2, Ifnk*
rs6287606	4	9.388400	18.12	4.987	*Lxw1, Aaom1*
rs13479014	6	62.998	125.05	4.538	*Cypr5,Dbm1, Ibdq2, Ltbr*
rs6389420	6	63.737	127.04-	4.440	*Cd4,Cd69, Cypr5, Ibdq2, Il17ra*
rs3681620	6	64.536	128.00-	3.705	//
CEL.6_130920075	6	65.362	130.19	3.786	*Cia3, Foxj2, Foxm1, Irak2*
rs6339546	6	67.03	133.97	3.563	//

The table presents the SNPs that showed genotype/phenotype overlapping for *AIP 2* QTLs and *AIP 5* QTL and $CD4^+$ $IL17^+$ on Chr.6 with the highest -log P value as well as their position. QTL, quantitative trait loci; SNP, single nucleotide polymorphism; Mbp, mega base pair; cM, centimorgan.

a. Position according to the NCBI Build 37

b. Position retrieved from Ensembl. (http://www.ensembl.org/Mus_musculus/Location)

c. -log P value of the association.

The most important findings in these regions in chromosomes 2 and 6 are listed in Table 3.12. Interestingly, these two AIP QTLs that linked to $CD4^+IL17^+$ phenotype colocallized with the previously identified autoimmune QTLs, namely *Cypr5*, *Cia3* and *Ibdq2*. Some genes in these intervals such as lymphotoxin B receptor gene (*Ltbr)*, interleukin 17 receptor A gene (*Il17ra*), *Cd4* antigen and *Cd69* antigen might be suggested as candidate genes for this phenotype.

Table 3.12 QTLs and genes next to $CD4^+$ $IL17^+$ phenotype` peaks on chromosomes 4 and 6

Symbol [a]	Type	Name	Chr.[b]	Position (bp) [c]
Nbwa2	QTL	NZB and NZW autoimmunity 2	4	34892515-34892635
Ifnk	gene	interferon kappa	4	35152056- 35154005
Lxw1	QTL	lupus BXSB x NZW 1	4	25683240-25683429
Aaom1	QTL	autoimmune aoritis in MRL mice 1	4	24322299-46332776
Cypr5	QTL	cytokine production 5 QTL	6	127838217-127838360
Dbm1	QTL	diabetes modifier 1 QTL	6	78629504-121115387
Ibdq2	QTL	inflammatory bowel disease QTL 2	6	106005405-120916853
Ltbr	gene	lymphotoxin B receptor gene	6	125306571-125313885
Cd4	gene	CD4 antigen;	6	124864692- 124888221
Cd69	gene	CD69 antigen	6	129267325-129275369
Il17ra	gene	interleukin 17 receptor A	6	120463197-120483727
Cia3	QTL	Collagen-induced arthritis QTL 3	6	113347325-113347522
Foxj2	gene	forkhead box J2	6	122819914-122845366
Foxm1	gene	forkhead box M1	6	128362967-128375893
Irak2	gene	interleukin-1 receptor- associated kinase 2	6	113638467-113695009

Chr.,chromosome; bp, base pair.

a. SNPs symbols and description retrieved from MGI database. (http://www.informatics.jax.org/searches/allele_form.shtml)

b. Chr.; chromosome.

c. Position retrieved from Ensembl. (http://www.ensembl.org/Mus_musculus/Location)

Interestingly, a single strong linkage to $CD4^+$ $IL17^+$ phenotype (as covariates in linkage mapping) was identified in region on Chr.18 (Figure 3.8) with the highest logarithmic P value in its peak for two SNPs:

- rs3658163 (Chr.18:68661314 bp, ~ 34.776 cM) (-log P value = 9.614). One of the QTLs in this region is T helper cell response (*Thcr*)[9] QTL (at position 44997537-6864706 bp), which controls NKT cell homeostasis and T helper cell differentiation in murine (Zhang et al., 2003; Zhang et al., 2006).

[9] (http://www.informatics.jax.org/marker/MGI:3036489)

- rs3691542 (Chr.18:66474868 bp, ~ 33.864 cM) (-log P value = 9.337). A protein coding gene, interleukin 17B (*Il17b*)[10] (Chr. 18: 61687935-61692537 bp) is located also in this region. The protein encoded by this gene is a T cell-derived cytokine that shares sequence similarity with IL17 (Shi et al., 2000).

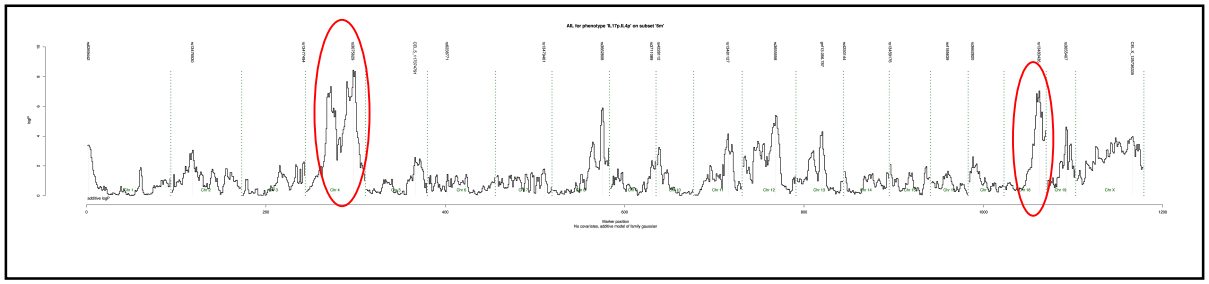

Figure 3.8 Whole-genome linkage map for AIP and $CD4^+$ $IL17^+$ as covariate in G4 mice

Logarithmic P values are presented in relation to positions of the marker loci along the chromosome and show relationships between QTLs and phenotype. The x-axis represents position in Mbp and the y-axis shows -log P value of the association.

[10] (http://www.informatics.jax.org/marker/MGI:1928397)

4 Discussion

Autoimmune pancreatitis is a rare disease that primarily involves the pancreatic duct bile ducts with characteristic histopathological changes in the tissue (Adarichev, Nesterovitch et al., 2003). Some of the typical histopathological features of AIP are a storiform fibrosis, dense periductal and diffuse lymphoplasmacellular infiltrates, obliterative periductal fibrosis and the presence of numerous CD4 and CD8 lymphocytes (Klimstra & Adsay, 2004; Okazaki et al., 2000; Zamboni et al., 2004).

Since the term "Autoimmune pancreatitis" was introduced (Yoshida et al., 1995), the field of AIP is extremely evolving. This relatively new entity has at least two subtypes: (a) lymphoplasmacytic sclerosing pancreatitis (LPSP; AIP type 1) with presence of IgG4-posivite cells, (b) idiopathic duct-centric chronic pancreatitis (IDCP; AIP type 2) or AIP with supposed granulocyte-epithelial lesions (GEL) (Chari et al., 2010; Okazaki et al., 2010; Zamboni et al., 2004). Although AIP type 2 shares some common features with AIP type 1, i.e. obstructive jaundice, diffuse pancreatic enlargement on imaging studies and the excellent steroid responsiveness, they are considered to be two distinct clinicopathological entities (Kamisawa, Funata, Hayashi, Eishi et al., 2003).

The pathoetiology of AIP is being extensively studied during the last decade. Some evidence suggests that in addition to ductal cells, acinar cells and their protein components are also targets of the immune-related inflammatory process that characterizes AIP. The most eminent finding are autoantibodies against trypsinogens (the cationic trypsinogen gene; *PRSS1* and the anionic trypsinogen; *PRSS2*) (Gao et al., 2013; Witt et al., 2006), trypsin inhibitor *PSTI* (Pancreatic secretory trypsin inhibitor) (Asada et al., 2006) and autoantibodies against ubiquitin subunit (UBR2; a particular acinar protein) (Frulloni et al., 2009). Using immunoassay and gene expression analysis, Löhr et al. detected autoantibodies against trypsinogen in AIP patients, which are useful to identify acinar cells and their secretory enzymes as a key target of the inflammatory process (Löhr et al., 2010). Beside the histopathological features of AIP, elevated autoantibodies against trypsinogens (*PRSS1* and *PRSS2*) and trypsin inhibitor PSTI (Asada et al., 2006) in AIP patients may facilitate clinical diagnosis of AIP and help to further distinguish it from non-AIP CP. Although an association between *PRSS1* gene mutations and AIP has been reported for the first time (Gao et al., 2013), it is possible to hypothesis that AIP is a separate disease entity with its own susceptibility genes and that the genetic basis of AIP differs from non-AIP forms of CP.

4.1 Four-ways Autoimmune-prone Advanced Intercross Line (AIL) Progeny

The most important goal of this study was to generate an outbred intercross mouse line, a four-ways autoimmune-prone advanced intercross line (AIL), the progeny of which represents a large family with higher incidence of common autoimmune diseases. Therefore, we took the advantage of the MRL/Mp mouse model of AIP (established by Kanno et al), the lupus-prone NZM2410/J strain, the arthritis-prone BXD2/TyJ mice and CAST/EiJ to generate F1 hybrids and future generations with high levels of heterozygosity and to identify genes contributing to autoimmune diseases susceptibility using QTLs mapping. This part of the project had started in 2008 at the University of Rostock and then continued in Lübeck at the UKSH. The sixteenth generation (G16) of this line is available now. Although we focused particularly on the genetic basis of AIP and its pathophysiology in this study, it should be mentioned that our established four-way autoimmune-prone AIL mice were also used in parallel in other studies to identify loci contributing to arthritis, epidermolysis bullosa acquisita and systemic lupus erythematosus. Taken together, data from G4 mice that developed autoimmune diseases led to the identification of a number of previously unknown QTLs that are located on different murine chromosomes (Asghari et al., 2011; Ludwig et al., 2012; Ranea et al., 2013). Ongoing fine mapping studies at the level of the G16 aim at reducing the size of the QTL and will be completed in 2014.

4.2 Development of AIP

In order to monitor development of spontaneous AIP in parental and G4 mice, histological and immunohistochemical analyses of pancreas sections were performed. Histopathological examinations clearly showed that the G4 mice spontaneously developed pancreatic lesions with an incidence of 7.4 % in male and 17.6 % in female mice at the age of 6 months (Table 3.1). These results match those observed in earlier studies, which specified as a typical feature of the MRL/Mp mouse model of AIP that the incidence and severity of the disease are significantly higher in female mice. The onset of the disease is earlier than in male individuals (Kanno et al., 1992; Qu et al., 2002; Sorg et al., 2008; Yu, Wester-Rosenlöf et al., 2009). The histological changes reported in the present study are also in agreement with previous studies, which indicated that histopathological changes, specifically lymphoplasmacytic infiltrates were more severe in female mice then male individuals (Figure 3.1). Similar results were observed for organ destruction as well.

It has previously been thought that $CD4^+$ T lymphocytes (compared with $CD8^+$ T lymphocytes) are critical in mediating AIP pathogenesis (Klöppel et al., 2003; Zandieh & Byrne, 2007). This interpretation contrasts with that of Li et al. (2011) who reported a case of AIP with a significant $CD8^+$ infiltration and an absence of $CD4^+$ T lymphocytes in the pancreas and surrounding organs, including the duodenum (Li et al., 2011). On the other hand, several studies have shown that infiltrating cells in AIP cases predominantly consisted of $CD4^+$ T lymphocytes with few detectable $CD8^+$ T lymphocytes and B lymphocytes (Kim et al., 2004; Klöppel et al., 2003). The results of the current study also support the findings of Sorg et al. in 2008, who have noted the importance of infiltrated $CD4^+$ and $CD8^+$ T lymphocytes, plasma cells, granulocytes and macrophages (Sorg et al., 2008) for characterizing AIP in MRL/Mp mice.

Pancreatitis in G4 mice was characterized by inflammatory cell infiltrates with destruction of acinar cells and replacement by fibrous tissue. Characterization of the inflammatory cells demonstrated that a high percentage of organ-infiltrated inflammatory cells were $CD4^+$ and $CD8^+$ T lymphocytes (Figure 3.2). However, granulocytes and macrophages were also detectable, as has previously reported by Sorg et al. (Sorg et al., 2008). Moreover, immunohistochemical examination revealed that $CD4^+$ T cells and macrophages mediate pancreatic inflammatory lesions. The histopathology of the pancreatitis in this animal model mimics human autoimmune pancreatitis of subtype 1.

4.3 QTL Analysis

Until recently, the genetic basis of AIP has not been systematically investigated. This might be due to the limited availability of suitable animal models for the disease. Despite low incidence of the disease, identification of susceptible and resistance genes of AIP could be important to achieve new diagnostic markers and alternative treatment strategies of human AIP and for further studying the immunopathogenesis of this disease.

In order to understand the genetic basis of AIP, a full genome scan was performed for 330 G4 four-way AIL mice using a set of 1400 SNPs to identify novel QTLs linked to AIP. The first genotype/phenotype association analysis that was performed in these mice led to the mapping of five AIP -associated QTLs (Asghari et al., 2011). The following five QTLs, so called *AIP1-5*, were identified with support confidence intervals of ~ 12 cM, which still contain hundreds of genes. *AIP1-5* OTLs were mapped to mouse chromosomes 2, 4 (n=2), 5

and 6 with logarithmic P values varying from 3.2 to 5.4 (Table 3.2). It is interesting to note that all identified loci in this study overlap partially, but not completely, with known loci previously linked to arthritis (*Cia2*, *Cia4*) and lupus erythematosus, suggesting similarities between the primary susceptibility genes of AIP and other autoimmune diseases. On the other hand, in a parallel study in G4 mice, Ludwig et al. reported a maximum disease severity associated with QTLs of epidermolysis bullosa acquisita on chromosomes 1, 15 and 19, none of which overlapped with the newly identified QTLs of the AIP (Ludwig et al., 2012). The other five loci, contributing to arthritis, that were identified on chromosomes 3, 7, 13, 18, and X in G4 mice didn´t also overlap with AIP QTLs (Ranea et al., 2013). A possible explanation for this might be that AIP is a separate disease entity with its own susceptibility genes, and that the genetic basis of AIP differs from other autoimmune diseases.

4.3.1 *AIP1* QTL and Its Candidate Genes

The newly identified *AIP1* QTL (Table 3.2) was mapped on chromosome 2 with confidential interval between 56.3 to 81.9 Mbp. Two peaks of this QTL, one at 64.9 Mbp (-log P value = 4.4) and other at 74.7 Mbp (-log P value = 4.3) (Table 3.4) are relatively close to the peak of the well known QTL susceptibility to experimental allergic encephalomyelitis susceptibility 21 (*EAE21*). *EAE21* is one of 10 identified QTLs that control susceptibility or clinical signs of experimental allergic encephalomyelitis (EAE) in the mouse genome (Butterfield et al., 2000). Multiple sclerosis (MS) is the most important inflammatory disease of the central nervous system (CNS), which involves autoimmune responses to myelin antigens. The role of T cells in the pathogenesis of the disease, particularly $CD4^+$ T cells that secrete IL17, has been recently studied in experimental autoimmune encephalomyelitis (EAE), an animal model for MS, as well as other AID (Fletcher et al., 2010). In a study that was performed by Butterfield et al., it has been suggested that the severity and location of CNS lesions in EAE are genetically controlled. Moreover, the genetic component controlling the character and severity of the lesions can also be influenced by sex (Butterfield et al., 2000).

There are a number of genes found in this locus (Table 3.3), some of which are close to the peaks of QTL. Despite having many potential candidate genes in this locus on chromosome 2, some of them are most likely the main susceptibility candidate genes. These genes are *HnRNP A3*, Nuclear factor erythroid derived 2 like 2, Sjogren 's syndrome antigen B and Ubiquitin protein ligase E3 component n-recognin 3 (*Ubr3*). These genes are involved in

pathways that play a significant role in the regulation of inflammatory process. Although a fine mapping of the QTLs still has to be accomplished, a first search for possible candidate genes was already performed. Therefore, we focused on the two QTLs with higher logarithmic P values (>4), which are located on chromosomes 2 and 6 (Table 3.3). The most promising findings in these two chromosomes including their position and SNPs next to the peaks of QTLs are summarized in Table 3.3. Subsequently, these potential candidate genes are discussed in detail in this chapter. Moreover, details of SNPs next to the peaks of AIP QTLs (*AIP1* and *AIP5* QTLs) are given in Table 3.4.

4.3.1.1 Heterogeneous Nuclear Ribonucleoprotein A3 Gene (*Hnrnpa3*)

The first candidate gene in this region (*AIP1* QTL) is *Hnrnpa3* (Chr2:75659261-75669407 bp, ~ 44.75 cM). Ribonucleoprotein particles of the Heterogeneous nuclear ribonucleoproteins (hnRNPs), which belong to the spliceosomal macromolecular group, are involved in various cellular functions, including precursor of mRNA processing (actively participating in pre-mRNA metabolism), transcriptional regulation and DNA recombination (He & Smith, 2009). For unknown reasons ribonucleoprotein particles (RNP) are frequently targeted by autoantibodies and thus play a significant role in a variety of human systemic autoimmune diseases. They are also associated with the pathophysiology of disease (Süleymanoğlu, 2003), which makes them valuable prognostic markers in terms of molecular epidemiology and pathogenesis of autoimmunity. Particularly, HnRNP A3 has been reported to bind to the 3′-UTR region of the cyclooxygenase-2 (Cok et al., 2004) and the tumour necrosis factor-α gene (Rousseau et al., 2002), suggesting a link to the regulation of inflammatory responses. Some members of the hnRNP family are involved in systemic autoimmune diseases such as lupus erythematosus and represent important autoantigens (Fritsch-Stork et al., 2006; Süleymanoğlu, 2003). Although very little is known from the literature regarding the specific role of *Hnrnpa3* or its correlation with AIP, the present findings seem to be consistent with other research that has implicated the Hnrnpa2 protein in lupus erythematosus (Fritsch-Stork et al., 2006; Süleymanoğlu, 2003). Therefore, it can be suggested that *Hnrnpa3* (as a major T -cell autoantigen) may be directly involved in the development of AIP.

4.3.1.2 Nuclear Factor, Erythroid Derived 2, Like 2 Gene (*Nfe2l2*; *Nrf2*)

Nuclear factor erythroid derived 2 like 2 (*Nfe2l2* or *Nrf2*) is the next candidate gene in this region (Chr2: 75675513-75704641 bp, ~ 44.75 cM). The corresponding protein is the main transcription factor of the antioxidant response pathway. It has been reported that *Nrf2*-null mice develop a lupus-like autoimmune syndrome, which is characterized by multiorgan inflammatory lesions with a marked female predominance, appearance of anti-double-stranded DNA antibodies and intravascular deposition of immunoglobulin complexes in blood vessels. Moreover, an enhanced proliferative response of $CD4^+$ T cells with altered ratios of $CD4^+$ and $CD8^+$ cells and increased oxidative lesions in tissues of *Nrf2*-null mice have been observed (Ma et al., 2006; Yoh et al., 2001).

Regarding the biological function of *Nrf2*, Singh et al. stated that *Nrf2* binds to the antioxidant response element (ARE) in the regulatory regions of multiple genes involved in the antioxidant defence system (Singh, Vrishni, Singh, Rahman, & Kakkar, 2010). Briefly, this function initiates in response to electrophilic and oxidative stress. Following its activation, *Nrf2* binds to ARE, which consequently upregulates a series of antioxidant and detoxifying genes. Thus silencing of *Nrf2* may open a new window in treatment of oxidative stress-mediated diseases such as cancer. It has been shown that significant antioxidant functions, which are involved in the control of peripheral lymphocyte homeostasis and autoimmune surveillance, can be mediated by *Nrf2* (Ma et al., 2006; Yoh et al., 2001).

4.3.1.3 Sjogren´s Syndrome Antigen B Gene (*Ssb*)

Sjogren syndrome antigen B (*Ssb*) (Chr2:69861562-69871786 bp, ~40.95 cM) is another candidate gene that located in the same genomic region as *AIP 1* QTL (Table 3.2) *SSb* is an important gene associated with Sjogren's syndrome. A common finding in patients with Sjogren´s syndromeare is antinuclear antibodies, which are directed against ribonucleoprotein SSB (autoantigen La) (Routsias & Tzioufas, 2010). The potential link between AIP susceptibility and SSB is interesting since sialadenitis represents the most frequent association of AIP (Klöppel et al., 2003). However, it should be noted that human sialadenitis associated with AIP differs from Sjogren´s syndrome in some important regards, i.e. lack of anti-SSA/SSB autoantibodies and prominent infiltration of IgG4-positive cells (Okazaki et al., 2010). Moreover, an association between autoantibodies to Ro/SSA and La/SSB with DR3,

DQA and *DQB* alleles has been reported in different studies (Bolstad & Jonsson, 2002; Fei et al., 1991).

4.3.1.4 Ubiquitin Protein Ligase E3 Component n-Recognin 3 Gene (*Ubr3*)

The last candidate gene in this region is ubiquitin protein ligase E3 component n-3 Recognin (*UBR3*). *Ubr3* is a protein-coding gene that belongs to the Ubr1 family of E3 ubiquitin-protein ligases, acting as a component of the N-end rule pathway. Deficiency of the related protein Ubr1 has been shown to cause Johnson-Blizzard syndrome, an autosomal-recessive disorder associated with intrauterine-onset destructive pancreatitis, congenital exocrine pancreatic insufficiency, malformations and mental retardation (Zenker et al., 2005). Furthermore, an autoantibody against the plasminogen-binding protein of Helicobacter pylori, which is present in 95% of AIP patients, has been suggested to cross-react with UBR2, an enzyme highly expressed in pancreatic acinar cells (Frulloni et al., 2009). As noted above, similarity between UBR3 and UBR2, which acts as a potential autoantigen in AIP and association with Sjogren's syndrome, is of interest. Therefore, the role of UBR proteins in the pathogenesis of the CP and specifically AIP required further investigation. Follow-up studies in this field should include detection of autoantibodies and UBR3-reactive T-cells in AIP models and patients.

Since AIP is a complex disease controlled by many factors, it is reasonable to speculate that genes controlling other immune phenotypes, such as antibodies and T cell subsets, might affect AIP development. Thus, the four genes mentioned above could be considered as candidate genes for *AIP1* QTL.

4.3.2 *AIP5* QTL

The next AIP QTL, *AIP5* QTL displays a maximum -log P value (=5.4) and maps to CI between 111.2 to 133.9 Mbp (Table 3.2). As mentioned in Table 3.2, *AIP5* QTL also overlaps with Collagen-induced arthritis QTL 3 (*Cia3*), which is one of the two identified loci contributing to CIA severity (*Cia2* on chromosomes 2 and *Cia3* on Chr. 6) (McIndoe et al., 1999). In our previous study, *Cia3* was refined into a 5.6 Mb genomic region on mouse chromosome 6 and candidate genes were identified for this locus as well, which will help to find the susceptibility gene(s) controlling arthritis development within *Cia3* and its counterpart regions in rat and human genomes (Yu, Teng et al., 2009).

This QTL contains several genes, two of which are close to its peak marker (rs3695725 at 121.2 Mbp) and appear to be of particular interest as candidate genes. One of these genes is C-type lectin domain family 4, member a2 gene (*Clec4a2*; at 123.1 Mbp), which is approximately 1.9 Mbp away from the peak marker of this QTL and the other one is peroxisome proliferator-activated receptor gamma (*Pparg*; at 115.4 Mbp), which is approximately 5.8 Mbp far from the peak of this QTL (Table 3.3 & Table 3.4). It is interesting to note that these two genes participate in maintaining homeostasis of the immune system as well as regulation of cell growth, differentiation and inflammation. Therefore, they could be considered as putative candidate genes for *AIP5* QTL for further studies.

4.3.2.1 C-type Lectin Domain Family 4, Member a2 Gene (*Clec4a2*)

Clec4a2, also known as dendritic cell immunoreceptor (*DCIR*), is a C-type lectin receptor expressed mainly in dendritic cells (DCs). C-type lectins on dendritic cells can influence cellular immune responses by their functions as antigen uptake and signaling receptors (Bloem et al., 2014). Studies in DCIR-deficient mice suggest that this receptor is a negative regulator of DC expansion and has an important role in maintaining the homeostasis of the immune system, which was observed previously in collagen -induced arthritis animal model (Fujikado et al., 2008). Accordingly, an obviously exacerbated response to collagen-induced arthritis was observed in Dcir $^{-/-}$ mice. Recently, *DCIR* has also been implicated into the pathogenesis of human rheumatoid arthritis (Eklöw et al., 2008). Another recent work has also suggested that *DCIR* could be considered as a novel receptor for intravenous immunoglobulin (IVIg), which can mediate interaction of innate and adaptive immunity in tolerogenic responses (Massoud et al., 2013).

In current study, *AIP5* QTL (confidence interval of 111.2 to 133.9 Mbp) displays the highest logarithmic P value (-log P value =5.4) and contains the C type lectin domain family 4 member a2 (*Clec4a2*) (at 123.1 Mbp), which is located in a distance of 1.9 Mbp next to the peak marker of the QTL (rs3695724) (Table 3.4). *Clec4a2* is main susceptibility gene of *AIP5* QTL for several reasons. Firstly, it encodes a receptor protein of dendritic cells that has previously been implicated in autoimmune diseases such as Sjogren's syndrome. Second, the association with Sjogren's syndrome has been observed in AIP patients suggesting that they share the same genes that seem to be involved in multiple autoimmune disorders. In addition, the role of *DCIR* in interaction of innate and adaptive immunity in tolerogenic responses suggests this gene as promising gene for *AIP5* QTL.

4.3.2.2 Peroxisome Proliferator Activated Receptor Gamma Gene (*Pparg*, *PPARγ*)

The second candidate gene for *AIP5* QTL is Peroxisome proliferator-activated receptor gamma (*Pparg*), which is roughly 5.8 Mbp away from the peak of the QTL (Table 3.3). As a member of the nuclear receptor superfamily of ligand-dependent transcription factors, Peroxisome proliferator-activated receptor γ (*PPARγ*) is a key regulator of cell growth, differentiation, and inflammation in different tissues. Furthermore, some studies have implicated the role of *PPARγ* in the regulation of critical aspects of development and homeostasis, including adipocyte differentiation, glucose metabolism, cell cycle control, and monocyte/macrophage differentiation, development and function (Han et al., 2001; Lehmann et al., 1995; Spiegelman, 1998; Tontonoz et al., 1998). The ligands of *PPARγ*, such as thiazolidinedione derivatives, have been shown to exert therapeutic effects in experimental acute and chronic pancreatitis, as well as to inhibit pancreatic fibrosis by targeting pancreatic stellate cells (Hashimoto et al., 2003; Jaster et al., 2005; Shimizu et al., 2002; Van Westerloo et al., 2005).

The confidence interval of AIP, lupus and arthritis QTLs still contain hundreds of genes. Therefore, it is feasible that the genes associated with AIP could be different from those associated with lupus or arthritis. Nevertheless, it is also possible that the primary susceptibility genes for AIP will be shared with other autoimmune diseases. Similar observations were made in other genome-wide association studies of common autoimmune diseases in humans, e.g. rheumatoid arthritis, diabetes, inflammatory bowel diseases and type I diabetes. This could facilitate fine mapping, as mouse and in silico resources are available for those diseases. The G4 mice have been extensively phenotyped for signs of other autoimmune diseases, i.e. lupus and arthritis, and an analysis of the data to determine overlapping and disease-specific QTLs is currently in progress.

In the next step of this study, we focused on the other three AIP QTLs with -log P values of 3.2 ≥ 3.5, which are located on chromosomes 4 (n=2) and 5. Findings are summarised in Table 3.6.

4.3.3 *AIP2* QTL

AIP2 QTL was mapped on chromosome 4 (peak at 18 Mbp; CI: 7.1 to 32.3Mbp) (-log P value = 3.2). It can be seen from the data in Table 3.2 that this locus overlaps with two previously identified QTLs: Autoimmune aoritis in MRL mice 1 (*Aaom1*) and

Lupus BXSB ×NZW 1 (Lxw1). The association with autoimmune lupus susceptibility traits, a linkage to early anti-chromatin antibody production and antigen-specific antibody responses are outstanding finding for these two QTLs, which suggests that the common pathway may play a role in AIP and other autoimmune diseases.

4.3.3.1 Autoimmune Aoritis in MRL Mice 1 (*Aaom1*)

Aaom1 QTL is associated with autoimmune aoritis in MRL mice and is mapped on chromosome 4 at 13.3 cM. The interleukin 11 receptor (*IL11R*; ~12.7 cM) has been proposed as a possible candidate gene for Aaom1, which modulates antigen-specific antibody responses in vitro and in vivo (Yin et al., 1992). As MRL/lpr mice develop glomerulonephritis, sialoadenitis and arthritis spontaneously, most of the studies focused on this strain as a model of human lupus nephritis, rheumatoid arthritis, Sjögren syndrome as well as vasculitis. A study of recombinant inbred (RI) strains, MXH/lpr mice strains (MRL/lpr × C3H/lpr mice), suggested that systemic vasculitis in MRL/lpr mice is genetically controlled with cumulative effects of multiple gene loci with different tissue specificity (Nose et al., 2013). A linkage analysis on a population of F2 intercross mice (MRL × C3H) showed that MRL/MpJ as a susceptible parental strain develops spontaneous vasculitis whereas parental strain C3H/HeJ is resistant (Yin et al., 1992). It has been also reported that the Aaom1 locus in the MRL strain originated from an AKR/J or LG/J strain (Yamada et al., 2003).

4.3.3.2 Lupus BXSB x NZW 1 (*Lxw1*)

Lxw1 (lupus BSXB × NZW 1) QTL maps on mouse chromosome 4 (at 25,683,240-25,683,429 bp, ~ 6.5 cM) and has a linkage to early anti-chromatin antibody production at 6 months old mice. To identify QTLs associated with autoimmune lupus susceptibility traits, Kono et al performed a genome-wide association study by using (BXSB/Scr × NZW/LacScr) F2 female mice. Their result showed that the parental strain NZW/LacScr displays higher susceptibility for lupus-associated traits such as glomerulonephritis (GN) and arthritis. In addition, the BXSB-inherited chromosome 4 locus designated susceptibility at Lxw1, which was linked to early anti-chromatin antibody production (Hamano et al., 2006; Kono et al., 2003).

4.3.4 *AIP3* QTL

The next QTL, *AIP3* QTL, was mapped on Chr. 4 with two peaks at 82.4 and 103.6 Mbp (CI from 81.3 to 120.7 Mbp) (-log P value = 3.3) (Table 3.2 & Table 3.5). In this interval, many QTLs have been described affecting other autoimmune diseases like systemic lupus erythematosus susceptibility 2 (*Sle2*), Anti-dsDNA antibody production in NZM 1(*Adaz1*), Agnm2, Proteoglycan induced arthritis 27 (*Pgia27*) and Psoriasis-like skin disease severity 1(*Psds1*).

4.3.4.1 Systemic Lupus Erythematosus Susceptibility 2 (*Sle 2*)

In mice, the *Sle2* locus contains several loci that are mapped on chromosome 4, influencing the immune system and are associated with B cells hyperactivity (Mohan et al., 1997; Scatizzi et al., 2012). The *Sle2* locus contributes significantly to lupus pathogenesis by affecting B cell development (Xu et al., 2004; Xu et al., 2005). It is also linked to glomerulonephritis in the NZM2410 mouse. The NZM2410-derived *Sle2* lupus susceptibility locus induces abnormality in B-cell differentiation and afterward expansion of autoreactive B1a cells (Zeumer et al., 2011). It has been shown that *Sle2* cannot induce independently any autoimmune pathology. However, the synergy between *Sle2* and lpr leads to accelerated lymphadenopathy that largely target T cells (Xu et al., 2011). In addition, Sle 1, 2, 3 mice have been suggested as useful tools for studying the role of IFN in the pathogenesis of lupus (Sriram et al., 2012).

4.3.4.2 Anti-dsDNA Antibody Production in NZM 1(*Adaz1*)

Anti-dsDNA antibody production in NZM 1 (*Adaz1*) locus was mapped on chromosome 4 in NZM2328 mice which was suggestively linked to plasma levels of IgG anti-dsDNA autoantibodies (Waters et al., 2001). This locus stretches out in the region similar to *Sle2* in NZM2410. *Adaz1*, however, is derived from NZB, whereas *Sle2* is derived from NZW (Bagavant et al., 2004).

4.3.4.3 Autoimmune Glomerulonephritis in MRL 2 (*Agnm2*)

Agnm2 is a QTL associated with autoimmune glomerulonephritis in MRL2 and mapped on chromosome 2 at confidence interval ~ 45.76 cM (at 100423186-124575207 bp).

Glomerulonephritis (GN) is one of the most important diseases in SLE. Therefore, Miyazaki et al. performed a QTL mapping to identify genes associated with susceptibility to GN using (MRL/lpr × C3H/lpr) F2 mice. Three susceptibility loci on two chromosomes are designated loci of autoimmune GN in *MRL* mice*: Agnm1* at 19.8–24.5 cM, and Agnm2 at 49.6–57.6 cM on chromosome 4, and Agnm3 at 54.0–65.0 cM on chromosome 5. It has also been suggested that *Agnm1* and *Agnm2* could share two susceptibility loci to systemic vasculitis. In addition, recessive or semi-dominant GN susceptibility genes might exist in the MRL strain, a hypothesis that is based on the data of relative decrease in the incidence in the F1 or F2 mice (Miyazaki et al., 2005).

4.3.4.4 Proteoglycan Induced Arthritis 27 (*Pgia27*)

Studies in animal models of inflammatory arthritis have identified more than 20 loci that are either specific to this animal model or shared with other models or with RA in humans (Adarichev, Valdez et al., 2003; Barton et al., 2001; Besenyei et al., 2012). In various species, different chromosome regions are linked with some traits of arthritis (i.e. the severity, onset, susceptibility, the production of antibodies and cytokines) and are overlapping with several autoimmune diseases, suggesting that they share common genetic components (Bauer et al., 2004; Glant et al., 2004; Kamogawa et al., 2002). Studying the effect of sex on various traits in murine models of RA demonstrated differences in several features of arthritis between male and female (Adarichev, Nesterovitch et al., 2003; Tengstrand et al., 2004; Weyand et al., 1998). However, *Pgia27*, which is located on Chr.4 at position 35–45cM, is prominent in male and is associated with the severity of disease. In a genome-wide association study that was carried out by Adarichev et al., all QTLs revealed different LOD scores or variation of the peak position regarding sexes. Interestingly, few QTLs were entirely sex-associated with both binary and onset QTLs higher in females than in males. In contrast, severity QTLs were more prominent in males than in females (Adarichev, Nesterovitch et al., 2003). It has been shown that in a murine animal model of RA, proteoglycan-induced arthritis (PGIA), severe arthritis is dependent on the production of IFN-γ (Doodes et al., 2010; Moudgil et al., 2011). Studying the role of IL17 in PGIA showed that IL17is not completely required for autoimmune arthritis and the production of other pro-inflammatory mediators is compensating for the loss of IL17 in PGIA (Doodes et al., 2008).

4.3.4.5 Psoriasis-like Skin Disease Severity 1(*Psds1*)

Psoriasis-like skin disease severity 1 (*Psds1*) locus is mapped to 42.5 cM on mouse chromosome 4 and shows significant linkage to skin disease severity. It has also a weak linkage to defective $CD4^+$ dendritic cell development. Two potential candidate genes have been found in this interval:interferon alpha/ interferon beta (*Ifna/Ifnb*) gene cluster (~ 42.6 cM) and *Jun* (~ 44.6 cM). The *c-Jun* proto-oncogene is a member of the Jun family of transcriptional regulatory proteins and may play a role in regulating the genomic response to growth factors. In the linkage analysis, which was performed to identify genetic modifiers of psoriasis-like skin disease, parental strain C57BL displays accelerated psoriasis-like skin disease due to increased Ifna/Ifnb responses. In contrast, parental strain BALB does not develop skin disease. Another locus is mapped to an interval between D4Mit204 (~ 61.9 cM) and D4Mit54 (~ 66 cM) and was named *Psds2* (psoriasis-like skin disease severity 2).It shows significant linkage to defective $CD4^+$ dendritic cell development (Arakura et al., 2007).

4.3.5 *AIP4* QTL

AIP4 QTL was mapped on Chr. 5 (CI 74.8 to 96.6 Mbp) (-log P value = 3.5) (Table 3.2 & Table 3.5) and displayed an overlapping with *Cdcs10* QTL, a QTL that is associated with colitis in interleukin-10-deficient (*Il10* $^{-/-}$) mice (Mähler et al., 2002). Experimental inflammatory bowel disease (IBD) in this mice can occur spontaneously or is induced by chemicals, cell transfer, pathogens, or genetic mutation (de Buhr et al., 2006; Hillhouse et al., 2011).

4.3.5.1 Cytokine Deficiency Colitis Susceptibility 10 (*Cdcs10*)

Genome screens for quantitative trait loci (QTLs) regulating colitis susceptibility identified *Cdcs10* as one of main effective QTLs on chromosomes 5 at position 47.29 cM in interleukin-10-deficient (*Il10* $^{-/-}$) mice (Mähler et al., 2002). Using the IL10-deficient mouse model, the first colitogenic QTL was identified and termed cytokine deficiency-induced colitis susceptibility 1 (*Cdcs1*) (Farmer et al., 2001). This quantitative trait locus and it`s subintervals were replicated in several studies, which is based on dysregulation of innate or adaptive immunity or pathogen control in animal models (Bleich et al., 2010; Buettner & Bleich, 2013). Moreover, the associations between 10 QTLs (*Cdcs1–10*, cytokine deficiency-induced colitis susceptibility 1–10) and colitis susceptibility in segregating populations of *Il10* $^{-/-}$ mice

have been reported (Farmer et al., 2001; Mähler et al., 2002). The severity of colitis in these mice depends on their genetic background (i.e. mice with C3H/HeJBir background develop more severe colitis than animals with C57BL/6J background) (Ermann et al., 2011; Mähler et al., 2002).

Taken together, in this new four-ways autoimmune-prone advanced intercross line (AIL) five new loci linked to AIP were identified. The strongest linkage was found for a region on mouse chromosome 6, which has been implicated in our previous study of collagen-induced arthritis (Yu, Teng et al., 2009). The other strong linkages were observed for loci on chromosomes 2, 4 and 5 that have formerly been observes in susceptibility to experimental allergic encephalomyelitis, systemic lupus erythematosus and CIA. These five AIP-linked QTLs colocalized with QTLs identified in mouse models for other autoimmune diseases (i.e. RA, EAE, SLE), suggesting that, at least in part, common genetic pathways may play roles in AIP and the other autoimmune diseases. Although the final targets for the autoimmune response in these diseases are diverse, the same gene products could complete the mechanism to break down tolerance to self-components. Furthermore, our results provide additional support for the hypothesis that QTLs controlling other autoimmune diseases might affect AIP development as well.

4.4 Pathogenesis of AIP in G4 Mice

The initial events triggering AIP remain unknown. Some hypotheses proposed that primarily infection of pancreatic cells by viral or infectious agent may initiate an immune-mediated processe, which is supported by several observations (Filippi et al., 2009; Filippi & von Herrath, 2008; Oldstone et al., 1991; Yanagisawa et al., 2011). The autoimmune mechanism of AIP is based on molecular mimicry between ubiquitin-protein ligase E3 component n-recognin 2 (*UBR2*) and an amino acid sequence of the plasminogen-binding protein (PBP) of Helicobacter pylori, or a homology between Helicobacter pylori virulence factor cag A (cytotoxin-associated gene A) and the carbonic anhydrase II (Frulloni et al., 2009; Guarneri et al., 2005). Another important finding in this context was the mechanism which was suggested for the virus-induced AIP mouse model (Watanabe et al., 2003). Cumulatively, these features provide further evidence that AIP is an immune-mediated inflammatory process.

However, the pathogenesis of AIP remains to be explored further. Recently, considerable attention has been directed to T helper cell-released pro-inflammatory cytokines

and their role (regulation and responses) in the pathogenesis of AIP. Despite of its simplicity, the concept of Th1/Th2 inequity has been used to interpret the pathogenesis of organ-specific autoimmune diseases (Adorini et al., 2002). It is believed that T helper imbalance, Th1 against Th2, is associated with the initiation of AIP (Zen & Nakanuma, 2011). Moreover, elevated Th1 responses in both pancreatic and extra-pancreatic lesions have been reported in AIP patients (Okazaki et al., 2000; Zen et al., 2007). Furthermore, a Th1 immune response has been considered to be predominant in type 1 AIP (Okazaki et al., 2000), and T helper 1 cell-released pro-inflammatory cytokines have been implicated in the pathogenesis of autoimmune pancreatitis. On the other hand, it has been demonstrated that the number of regulatory T-cells (Tregs) are increased in tissue-resident lymphocyte populations and whole blood of patients with type 1 AIP (Zen et al., 2007). In contrast to earlier findings, however, recent studies provided data in support of a Th2 predominant immune response in IgG4-RD (Okazaki et al., 2013).

Differentiation of murine $CD4^+$ T cells into Th1 or Th2 or Th17 subsets is dependent upon many different factors. For instance, IFN-γ and IL12 initiate the differentiation of Th1 cells, whereas IL4 triggers the differentiation of Th2 cells. There is considerable evidence that show the importance of IFN-γ and IL17 in the development and progression of inflammatory and autoimmune diseases (AID) in both humans and mice. It has been also suggested that an autoimmune mechanism, relying on Th1 immune response, might be involved in AIP. IFN-γ is a pro-inflammatory cytokine released by Th1 cell and plays a role in the pathogenesis of autoimmune pancreatitis. It has been shown that IFN-γ treated AIP-prone MRL/Mp mice developed more severe AIP (Fitzner et al., 2009).

Based on all these data, it is reasonable to expect that a QTL controlling T-helper cell differentiation may also play a role in immune-mediated diseases, especially in AIP. Therefore, the results of our analysis that show the overlap of several QTLs controlling immunological traits with AIP QTLs may be important in understanding the role of T helper cells in the pathogenesis of AIP.

4.4.1 *AIP1* QTL and Immune Cell Phenotypes

To examine whether AIP QTLs overlap with QTLs controlling Tregs cell development, we examined the association between AIP QTLs and $CD4^+FoxP3^+$ and $CD8^+FoxP3^+$

phenotypes. This revealed the potential pathway in which the susceptibility genes inside these QTLs might be involved.

Figure 3.5 and Table 3.9 provide the largest data set from *AIP1* QTL. The most interesting finding was a complete overlapping between all 12 markers (SNPs) associated with AIP in this region and $CD4^+$ $FoxP3^+$ and $CD8^+$ $FoxP3^+$ phenotypes with high logarithmic P values varying from 3.2 to 7.11. The peak region of *AIP1* QTL with the highest -log P value (=7.11) for $CD4^+$ $FoxP3^+$ phenotype overlaps with the previously identified QTLs associated with arthritis (*Cia2* and *Cia4*, two QTLs that controlling severity and onset of the disease) (Bauer et al., 2004) and the murine models of multiple sclerosis (*Eae21*and *Eae33*) (Butterfield et al., 2000; Peláez et al., 2005). In contrast, QTLs for both these phenotypes do not overlap with other AIP QTLs on chromosomes 4, 5 and 6. These results are consistent with those of other studies, and suggest that the susceptibility gene(s) inside these QTLs are affecting the AID clinical phenotypes (Bailey et al., 2007; Yu, Teng et al., 2009). This also supports a possible role for Tregs in the initiation of the disease. Moreover, the findings of the current study correspond to observations made by Koyabu et al. who reported a correlation between an early development of pancreatitis with an abundant infiltration of Foxp3-positive cells in MRL/Mp mice treated with poly I:C. This is highlighting a possibility that Tregs may be involved in the development of pancreatitis in these mice (Koyabu et al., 2013).

Interestingly, a strong linkage was observed for this locus (rs6378343, 6:63823084bp, ~ 32.20cM) with $CD8^+$ $FoxP3^+$ phenotype (-log P value = 4.9) (Figure 3.5 B). This locus overlaps with *Cia6*, collagen-induced arthritis QTL 6 (Popovic et al., 2008), a QTL that shows an association to the region encoding the *Tcr* beta chain (H.-T. Yang et al., 1999). Four genes were mapped in this region between 30.81 and 32.14 cM. These genes are involved in a co-stimulatory pathway that plays a key role in regulating the inflammatory process interaction between APC and T cells. These genes are CD8 antigen alpha chain (*Cd8a*), CD8 antigen beta chain 1 (*Cd8b1*), interleukin 23 receptor (*Il23r*) and interleukin 12 receptor, beta 2 subunit (*Il12rb2*).

Interleukin 23 receptor (*Il23r*) encodes a protein that serves as a subunit of the receptor for IL23A/IL23 and couples with the receptor molecule IL12R beta1. Both of these receptors are required for IL23A signaling. Fundamentally, this receptor protein binds to Janus kinase 2 (*JAK2*), and the transcription activator *STAT3* (Parham et al., 2002) that is required for the differentiation of T helper17 cells (Bettelli et al., 2007; X. O. Yang et al., 2007) that have been implicated in a variety of autoimmune diseases. Moreover, it has been reported that IL12

receptor beta 2 subunit plays a role the inflammatory response and host defense (Rodgers & Miller, 2012).

Interestingly, one of the most strongly associated loci with Foxp3$^+$ phenotypes was mapped on Chr. 7 (rs3699086, ~ 52.28cM) with maximum -log P value (=7.38) (Figure 3.5). This locus overlaps with "IL4 producing potential QTL **(***Lat***)",** which is essential to T cell activation (W. Zhang et al., 1998). Putative candidate genes found in the region of the peak marker may have an influence on the inflammatory process of an autoimmune disease. These genes are IL4 receptor alpha gene **(***Il4ra***)**, interleukin 18 binding protein **(***Il18bp***)** and immunoglobulin super family member 6 **(***Igsf6***)**, which map to the inflammatory bowel disease 1 locus (Bates et al., 2000).

Il4ra encodes a protein, alpha chain of the IL4 receptor, which can bind IL4 to promote differentiation of Th2 cells (Sharma et al., 2013). In 2006, a single significant QTL on chromosome 7 has been identified, controlling the potential production of IL4 in SLE model of NZB, BXSB and related mouse strains. *Il4Rα* gene, as a putative gene in this region, seems to be interesting due to identical sequence between NZB and BXSB strains, and eight amino acid differences between NZB and the NZW strains. However, a limited potential of IL4 production has been detected in both SLE-prone NZB and BXSB mice, whereas disease-free NZW mice had a high potential and (NZB × NZW) F1 and (NZW × BXSB) F1 are in between both of them. Therefore, it is logical to propose that the NZW-type polymorphism controls a high potential and that the NZB/BXSB-type polymorphism controls a low potential for IL4 production by T cells. Thus, the low IL4-producing phenotype appears to predispose to SLE in BXSB, but not in NZB-related strains, suggesting that the role of IL4 in the pathogenesis may differ between certain subsets of SLE, even if they show similar disease phenotypes (Shiroiwa et al., 2007).

Interleukin 18 binding protein (*Il18bp*) a novel modulator of the Th1 cytokine response, encodes a protein, which is an inhibitor of the pro-inflammatory cytokine IL18 (Novick et al., 1999). This protein is regularly expressed and secreted in mononuclear cells, binds to IL18, prevents the binding of IL18 to its receptor, and thus inhibits IL18-induced IFN-gamma production.

The other locus maps on Chr.9 (rs3700596, ~ 43.49 cM) and shows a significant association for two following phenotypes: CD4$^+$ FoxP3$^+$ (-log P value = 8.975), CD8$^+$ FoxP3$^+$ (-log P value = 5.63). This interval contains T lymphocyte fraction gene (*Tlf*)

(~ 52.24 cM), which is associated with autoimmune diabetes and appears to be a putative gene for this loci (Pearce, 1998).

It is interesting to note that all 12 SNPs in the region controlling AIP susceptibility (*AIP1* QTL) showed a strong linkage to $CD8^+ CD69^+$ phenotype with logarithmic P value of 3.31 to 6.46 (Table 3.9 and Figure 3.5). CD69 is a marker of early T cell activation and expressed on activated lymphocytes. Its selective expression in inflammatory infiltrates suggests that it plays a role in the pathogenesis of inflammatory diseases (Hasegawa et al., 2013). Respectively, the association between the leukocyte activation antigen CD69 and T cell differentiation towards Th1 or Th17 has been reported in several studies (Dorfman & Shahsafaei, 2002; Martin et al., 2010). Taken together, our observation supports the conclusion that genetic factors contribute significantly to an inflammatory process in G4 mice. Particularly, this part of our results confirms the role of the early activation receptor CD69 to modulate T-cell differentiation as a part of the immune inflammatory response in this progeny. To investigate the immunological pathways more thoroughly, the influence of AIP QTLs on the T helper cells differentiation towards Th1, Th2 and Th17 and a possible linkage between AIP QTLs and T helper subtype markers were verified.

4.4.2 Effect of AIP QTLs on Th1/Th2-type Cellular Immune Response

It has been shown that autoimmune-related pancreatitis is associated with autoantibodies and a Th1/Th2-type cellular immune response (Okazaki et al., 2000). Qu and coworkers demonstrated that TNF-α levels are increased in the serum of patients with pancreatitis as well as in animal models of pancreatitis (Qu et al., 2002). To determine the effects of IFN-γ, Fitzner et al. applied the cytokine directly to AIP-prone MRL/Mp mice. According to their results, IFN-γ accelerates and aggravates AIP in MRL/Mp mice (Fitzner et al., 2009). Therefore, to examine the effects of Th1/Th2 cellular immune response in AIP, the overlap between AIP QTLs and $CD4^+$ IFN-γ^+ and $CD4^+$ $IL4^+$ phenotypes as covariates was analysed, thus providing information about possible mechanisms underlying AIP QTLs function.

To identify quantitative trait loci controlling Th1/Th2 phenotypes in G4 mice, we examined five AIP QTLs by investigating the association between them and the immunological phenotypes. *AIP2* QTL was strongly linked to $CD4^+$ IFN-γ^+ (Th1) phenotype in its peak markers, as indicated by one peak on Chr. 4 at 9.39 cM (-log P value = 9.012) and another peak at 8.34 cM (-log P value = 5.132) (Figure 3.6). Previous studies also identified

Lxw1 and *Aaom1*, and *Nbwa2* (NZB and NZW autoimmunity 2 QTL) QTLs in this region that control clinical traits in arthritis and lupus animal models. This QTL contains the interferon kappa gene (*Ifn-κ*), which encodes a protein that is expressed at low levels in peritoneal macrophages. Its expression is up-regulated by dsRNA and IFN-γ. IFN-κ belongs to a recently identified subclass of type I IFNs (LaFleur et al., 2001). The murine type I interferon (*Ifn-kappa*) is expressed in the pancreatic islets and induces diabetes in mice (Vassileva et al., 2003). Overexpression of murine *Ifn-κ* in the β cells of the pancreas of transgenic mice induces development of diabetes that is histologically characterized by inflammatory infiltrates with corresponding destruction of β cells (Vassileva et al., 2003).

Interestingly, the same QTL, *AIP2* QTL, displays association with $CD4^+$ $IL17^+$ (Th17) phenotype (Figure 3.8) for both peak markers (mCV22939387 at position 8.341cM (-log P value = 4.609) and rs6287606 at position 9.388 cM (-log P value = 4.987). This finding seems to be consistent with previous reports which suggested that T helper inequity is associated with the initiation of AIP (Zen & Nakanuma, 2011).

4.4.3 T helper 17 (Th17) and AIP

It is interesting to address whether these newly identified AIP QTLs controlling immunological traits also affect the AIP development. The role of T helper 17 (Th17) and regulatory T cells (Tregs) in human organ transplantation and autoimmune diseases has been studied (Afzali et al., 2007) in detail. It has also been demonstrated that Th17 cells significantly contribute to the pathology of autoimmune diseases (Afzali et al., 2007; Kim et al., 2013). For example, in their study, Kim et al. detected elevated levels of Th17 cells, which are associated with disease activity in patients with rheumatoid arthritis (Kim et al., 2013).

In order to verify the linkage between AIP QTLs and immunological traits, and identify possible mechanisms underlying QTL function, a genotype/phenotype overlap analysis was performed in this study. The results indicate that in *AIP5* QTL region, out of 16 SNPs with a high logarithmic P values for AIP, five SNPs displayed overlap with $CD4^+$ $IL17^+$ phenotype. In *AIP2* QTL region, only 2 SNPs out of 5 SNPs (linked to AIP) showed overlap with this phenotype (Figure 3.8 & Table 3.10).

The most important findings in these regions are listed in Table 3.11. Interestingly, these QTLs (*AIP2* and *AIP5* QTLs) also overlap with previously identified autoimmunity QTLs, i.e. *Cypr5*; cytokine production 5 QTL, *Cia3* and inflammatory bowel disease QTL 2 (*Ibdq2*).

Some genes in this interval, lymphotoxin B receptor gene (*Ltbr*); interleukin 17 receptor A gene (*Il17ra***)**, *Cd4* antigen and *Cd69* antigen, seem to serve as possible candidate genes for this phenotype.

Probably, a part of these findings corroborates the ideas of Seleznik et al. who detected high messenger RNA levels of lymphotoxin (*LT*) α and β in pancreatic tissues from patients with AIP, compared with controls. The authors suggested that overexpression of *LT* αβ in acinar cells of the mouse leads to an AIP-like illness (Seleznik et al., 2012). As mentioned above, *AIP5* QTL contains lymphotoxin B receptor gene (*Ltbr*), which encodes a protein that is a member of tumor necrosis factor family (TNF). Pancreatic acinar cells produce, release, and respond to TNFα, a member of the TNF family, which regulates apoptosis in isolated pancreatic acini also in experimental pancreatitis (Bhatia et al., 2000; Gukovskaya et al., 1997).

Definitely, the most interesting finding in this analysis was a single strong linkage to $CD4^+$ $IL17^+$ phenotype for region (quantitative trait locus) that was identified on Chr.18 with the highest -log P value >9 (Figure 3.8) in its peak for two SNPs. The first peak marker (rs3658163 at 68661314 bp) for this locus (~34.776 cM) displayed a strong linkage (-log P value = 9.614) (Figure 3.8). Interestingly, this locus showed colocalization with T helper cell response QTL (*Thcr*) (at position 44997537-6864706 bp), which controls NKT cell homeostasis and T helper cell differentiation in mice (Zhang et al., 2003; Zhang et al., 2006). Several recent genetic studies showed that *Thcr* QTL also overlaps with previously identified murine systemic lupus erythematosus susceptibility loci (Vyse & Kotzin, 1998) and loci that are associated with murine experimental autoimmune encephalomyelitis (EAE) (Baker et al., 1995).

The other peak of this QTL (-log P value = 9.337) mapped to the region (rs3691542, at 66474868 bp, ~ cM 33.86) (Figure 3.8) involves interleukin 17B gene (*Il17b*) (Chr. 18: 61687935-61692537 bp). The protein encoded by this gene is a T cell-derived cytokine that shares sequence similarity with IL17 (Shi et al., 2000).

In addition, linkage studies of autoimmune diseases such as insulin-dependent diabetes mellitus, rheumatoid arthritis, systemic lupus erythematosus have identified susceptibility loci on human chromosome 18 in the region, which are homologous with the same region on mouse chromosome 18 (Cornélis et al., 1998; Merriman et al., 1998; Shai et al., 1999).

All together, this study has identified several QTLs associated with AIP clinical phenotypes, thus illustrating the underlying complexity of autoimmune diseases. Since AIP is a complex disease influenced by many factors, it is reasonable to speculate that genes controlling other immune phenotypes might affect AIP development. On the other hand, these newly identified QTLs co-localize with other known autoimmunity QTLs, suggesting common genetic components. Simultaneously, these evidences are providing essential information on the pathogenesis of AIP. Our results confirm the findings of a great deal of previous work performed in this field. Further research can be performed to identify the disease-responsible genes.

5 Conclusions

Recent advances in molecular and genetic methodologies should further confirm our understanding of AIP as a complex disease. The use of experimental animal models of AIP initiated a considerable progress towards understanding the pathophysiology of the disease. This study identified the main genetic loci (QTLs) controlling AIP. As in other autoimmune diseases, we expected AIP to be polygenic with a strong contribution of a small number of genes and probably weak contribution of large numbers of additional genes.

This is the first time such a study has been performed. Further intercrossing currently pursues fine mapping of the identified QTLs. Taken together, these insights into the pathogenesis of AIP will confidently lead to the identification of novel diagnostic and therapeutic targets.

6 Summary

Autoimmune pancreatitis (AIP) is a rare form of chronic pancreatitis that is characterised by infiltrates of inflammatory cells in the pancreas and, in severe cases, destruction of acinar tissue. T cell-mediated autoimmunity plays a predominant role in the pathogenesis of the disease, although the high prevalence of various autoantibodies indicates a humoral component of the autoimmune reaction as well. Despite being rare, AIP is clinically important since the disease is a differential diagnosis of pancreatic cancer and could be treated with steroids efficiently.

Using a mouse model of AIP, we have identified susceptible regions of the genome (quantitative trait loci; QTLs) which provide the first evidence for a genetic component of the disease. To identify quantitative trait loci of AIP, we established an advanced intercross line originating from three inbred mouse strains: MRL/MpJ (spontaneous development of AIP), BXD2 (susceptible to collagen-induced arthritis), NZM (model of Lupus erythematodes) and wild derived strain Cast (healthy controls).

This approach promises to identify both general autoimmune disease-associated genetic loci and further for AIP. Therefore, the fourth generation of outbred intercross mice was characterized phenotypically by scoring histopathological changes of the pancreas and genotyped with SNP arrays (Illumina, USA).

Five QTLs, regulating the severity of AIP were identified. Two of them mapped to chromosome 4 while one was mapped to chromosomes 2, 5 and 6 each. The QTL on chromosome 6 displays the highest logarithmic P value (-log P = 5.4) and contains the C type lectin domain family 4 member a2 near to peak SNP (rs3695724). The gene encodes a receptor protein of dendritic cells, which has previously been implicated in other autoimmune diseases such as Sjogren's syndrome. AIP candidate genes of other QTLs include heterogeneous nuclear ribonucleoprotein A3, nuclear factor (erythroid derived 2)-like2, Sjogren syndrome antigen B, and Ubiquitin protein ligase E3 component n-recognin 3 *(UBR3)*.

Moreover, we found *AIP* QTLs *1* and *5* overlapping with genetic loci controlling other immunological phenotypes in the same cross. The strongest evidence was suggested for the relative abundances of double positivity for $CD4^+$ $FoxP3^+$, $CD8^+$ $FoxP3^+$, $CD8^+$ $CD69^+$ and $CD4^+$ $IL17^+$ in mice splenocytes. The data suggest that QTL controlling Treg development or

T-helper cell differentiation could also modulate immune-mediated diseases, especially AIP, and that the susceptibility genes within these QTLs affect the autoimmune diseases clinical phenotypes.

A single strong linkage to $CD4^+$ $IL17^+$ phenotype (-log P >9, peak SNPs = rs3691542 & rs3658163) and a quantitative trait locus were identified on chromosome 18 in a region homologous to human chromosome 18. This locus with the highest logarithmic P values colocalizes with the T helper cell response (*Thcr*) QTL, which controls NKT cell homeostasis and T helper cell differentiation in mice. The locus contains the interleukin 17B gene (*Il17b*) which is a T cell-derived cytokine that shares sequence similarity with IL17.

This study identified the main loci controlling AIP in a mouse model of the disease. As in other autoimmune disorders, we could confirm the expectation of AIP to be polygenic with a strong contribution of a small number of genes (constrained by five dominant QTLs) and weak contribution of large numbers of additional genes. To the best of our knowledge, this is the first time such a study has been performed. The further fine mapping of the QTLs, in order to find candidate gene could be accessed using intercrossing more generation, which is now G16. These insights into the pathogenesis of AIP will hopefully lead to the identification of novel diagnostic and therapeutic targets.

7 Zusammenfassung

Die autoimmune Pankreatitis (AIP) ist eine seltene Form der chronischen Pankreatitis, die durch Infiltration von Entzündungszellen in die Bauchspeicheldrüse sowie in schweren Fällen auch durch die Zerstörung von Azinusgewebe gekennzeichnet ist. Die T-Zell-vermittelte Autoimmunität spielt eine bedeutende Rolle in der Pathogenese der Erkrankung, obwohl die hohe Prävalenz von verschiedenen Autoantikörpern auch eine humorale Komponente der Autoimmunreaktion belegt. Ungeachtet ihres seltenen Vorkommens ist die AIP klinisch wichtig, da sie eine Differentialdiagnose gegenüber dem Bauchspeicheldrüsenkrebs darstellt und effizient mit Steroiden behandelt werden kann.

Durch die Verwendung eines Maus-Modells für die AIP konnten Regionen des Genoms (Quantitative Trait Loci, QTLs), die mit der Anfälligkeit assoziiert sind, identifiziert werden und somit die ersten Beweise für eine genetische Komponente der Erkrankung aufgezeigt werden. Um QTLs für die AIP identifizieren zu können, wurde eine Vier-Linien-Kreuzung, genannt „advanced intercross line“ aus vier Mausstämmen generiert: MRL/MpJ (entwickeln eine spontane AIP), BXD2 (sind anfällig für die Kollagen-induzierte Arthritis), NZM (dienen als Modell für den Lupus erythematodes) und Cast (gesunde Kontrollen).

Dieser Ansatz verspricht die Identifizierung von Genombereichen, die sowohl allgemein mit Autoimmunerkrankungen als auch speziell mit der AIP assoziiert sind. Daher wurden die Mäuse aus der vierten Generation dieser Aufzucht-Kreuzung durch Bewertung der histopathologischen Veränderungen der Bauchspeicheldrüse phänotypisiert und mit einem SNP-Array (Illumina, USA) genotypisiert.

Fünf QTLs, die mit der Schwere der AIP korrelieren, wurden identifiziert. Zwei QTLs konnten auf Chromosom 4 lokalisiert werden, während die anderen sich auf den Chromosomen 2, 5 und 6 befinden. Der QTL auf Chromosom 6 wies den niedrigsten P-Wert (-log P = 5.4) auf und lokalisiert in der Spitzenregion (rs3695724) eine C-Typ Lektin-Domäne (*Clec4a2*). Diese kodiert für ein Rezeptorprotein dendritischer Zellen und wurde kürzlich mit anderen Autoimmunerkrankungen wie dem Sjögren-Syndrom in Verbindung gebracht. Weitere AIP- Kandidatengene der anderen QTLs sind das „heterogenous nuclear ribonucleoprotein A3 (*Hnrnpa3*)“, „nuclear factor (erythroid derived 2)-like2“, „Sjogren syndrome antigen B“, und „Ubiquitin protein ligase E3 component n-recognin 3 (*UBR3*)“.

Wir konnten zeigen, dass die identifizierten AIP QTLs 1 und 5 mit Genombereichen überlappen, welche mit immunologischen Phänotypen assoziiert sind. Die stärkste Evidenz konnten wir zur relativen Häufigkeit doppeltpositiver $CD4^+FoxP3^+$, $CD8^+FoxP3^+$, $CD8^+CD69^+$ und $CD4^+IL17^+$ Zellen aufzeigen. Diese Daten legen nahe, dass Immunerkrankungen, insbesondere die AIP, durch die QTL-gesteuerte Entwicklung von Treg-Zellen bzw. die T-Helferzell-Differenzierung moduliert werden können, und dass die Anfälligkeitsgene innerhalb dieser QTLs die klinische Ausprägung der Autoimmunkrankheiten beeinflussen können.

Eine starke Assoziation zum $IL17^+$ $CD4^+$ Phänotyp (-log P >9, Spitzen SNPs = rs3691542 & rs3658163) und ein QTL wurden außerdem auf Chromosom 18 identifiziert, in einer Region, die homolog zum humanen Chromosom 18 ist. Dieser Genort mit dem höchsten log(P)-Wert von > 9 ist kolokalisiert mit einem QTL für die T-Helferzellantwort (*Thcr*), der die NKT-Zell-Homöostase und die T-Helfer Zelldifferenzierung in Mäusen steuert. Dieser Lokus beinhaltet das Interleukin 17B-Gen (*Il17b*). Das entsprechende Protein ist ein T-Zell-Zytokin, welches Sequenzhomologien mit IL17 aufweist.

In dieser Studie wurden die wichtigsten Genombereiche identifiziert, welche im Mausmodell die AIP regulieren. Wie bei anderen Autoimmunerkrankungen konnten wir die Erwartung bestätigen, dass auch die AIP eine polygene Erkrankung ist, bei der eine kleine Anzahl von Genen (begrenzt durch fünf QTLs) mit großem Einfluss, sowie eine große Anzahl zusätzlicher Gene mit schwächerem Einfluss von Bedeutung sind. Nach unserem Kenntnisstand ist dies die erste Studie auf diesem Gebiet. Die Feinkartierung der identifizierten QTLs wird durch weitere Kreuzung fortgesetzt - die 16. Generation wurde gerade erreicht. Diese Einblicke in die Pathogenese der AIP werden hoffentlich zur Identifizierung neuer diagnostischer und therapeutischer Ziele führen.

8 References

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9 Appendix

Appendix 1. Immunological cell *markers* and mouse *CD markers for immunophenotyping*

Markers	Distribution
Basic T cells	
CD3	T cells
CD4	Tcells, Dcs, NK cells, Stem Cell/Precursor not Macrophage/Monocyte (Thymocyte subset, T subset, DCs)
CD8	T cells, DCs($CD8a^+$) (Thymocyte subset, T subset, DC subset, not fresh NK)*
CD45R (B220, $PTPRC^a$)	T Cells, B Cells, DCs, NK Cell, Stem Cell/Precursor, Macrophage/Monocyte (B, NK progenitors, LAK, T^{act}) *
CD4 or CD8 Memory/ Activated	
CD44	TCell, B Cell, Macrophage/Monocyte, Granulocyte, Erythrocyte, Endothelial Cell, Epithelial Cell (broad, memory T, not platelets, or hepatocytes) *
CD62L	T-Cell, B Cell, NK Cell, Macrophage/Monocyte, Granulocyte (B, T, monocyte, granulocyte, NK, thymocytes) *. Central memory T-lymphocytes, which have encountered antigen, express L-selectin to localize in secondary lymphoid organs
CD69	Very Early Activation Antigen, Nk cells, (T^{act}, B^{act}, NK^{act} , $granlocyte^{act}$, thymocytes, platelets) *
B cell Activation	
CD19	B Cell ,DCs, Stem Cell/Precursor (B, FDC, good B lineage marker,not plasma cells) *
CD69	Nk cells, (T^{act}, B^{act}, NK^{act} , $gran^{act}$, thymocytes, platelets)
CD86	T Cell, B Cell ,Dendritic Cell, Macrophage/Monocyte, Endothelial Cell (monocyte, B^{act}, NK^{act}, DCs)*
MHC Class II	APC, DCs, B cells
Dendritic Cell (DCs)	
CD11c	T Cell, DCs, NK Cell ,Stem Cell/Precursor, Macrophage/Monocyte, Granulocyte(DC, myeloid,NK, T subset)
PDCA-1	Plasmacytoid dendritic cells (PDC)
CD86	T Cell, B Cell ,DCs, Macrophage/Monocyte , Endothelial Cell (monocyte, B^{act}, NK^{act}, DCs)*
Regulatory T cells (Tregs)	
FoxP3 *	MHC class II restricted Foxp3-expressing $CD4^+CD25^+$ Tregs or MHC class I restricted $CD8^+$ Foxp3-expressing Tregs
T helpers	
IFN gamma	The signature cytokines of T helper1 cells, which was shown to assist Th1 development
IL4	T helper 2, activated $CD4^+$cells, memory $CD4^+$cells
IL17	T helper 17 cells

PTPRC; Protein tyrosine phosphatase receptor type C, DCs; Dendritic Cells, NK Cell; Natural killer cells, APC; antigen presenting cell, B act (also Tact. & NKact); activated B, T or NK cells, LAC; lymphokine-activated killer cell.

BD Bioscience Mouse CD Marker Poster and Handbook:
(http://www.ebioscience.com/resources/mouse-cdchart.htm)
(http://www.bdbiosciences.com/documents/BD_Reagents_CDMarkerMouse_Poster.pdf),
(http://www.bdbiosciences.com/documents/cd_marker_handbook.pdf),

*eBioscience Mouse CD & Other Cellular Antigens: (http://www.ebioscience.com/resources/mouse-cd-chart.htm#mouse-non-cd-chart)

9.1 Abbreviations

Aaom1	Autoimmune aoritis in MRL mice 1
AARDA	American Autoimmune Related Diseases Association
Adaz1	Anti-dsDNA antibody production in NZM 1
AFLP	Amplified fragment length polymorphism
Agnm2	Autoimmune glomerulonephritis in MRL 2
AID	Autoimmune diseases
AIL	Advanced intercross line
AIP	Autoimmune pancreatitis
ANA	Anti-nuclear antibody
Anti-ssDNA	Anti-single stranded DNA
Anti-dsDNA	Anti-double stranded DNA
APC-anti	Alkaline Phosphate *Conjugated antibody*
APC	Antigen presenting cell
ARE	Antioxidant response element
B10.Q	C57BL/10 strain with H2q haplotype of MHC
BCR	B cell receptor
bp	Base pair
BUN	Blood urea nitrogen
Cag A	Cytotoxin-associated gene A
CA-II	Carbonic anhydrase II
CCP	Cyclic citrullinated peptides
CD	Cluster of differentiation
Cd19	CD19 antigen
Cd4	CD4 antigen
Cd69	CD69 antigen
Cd8a	CD8 antigen, alpha chain
Cd8b1	CD8 antigen, beta chain 1
Cdcs10	Cytokine deficiency colitis susceptibility 10
cDNA	Complementary DNA
CFTR	Cystic Fibrosis Transmembrane Regulator
Chr.	Chromosome

CI	Confidential interval
CIA	Collagen-induced arthritis
Cia2	Collagen-induced arthritis QTL 2
Cia3	Collagen-induced arthritis QTL 3
Cia4	Collagen-induced arthritis QTL 4
Cia6	Collagen-induced arthritis QTL 6
Cia7	Collagen-induced arthritis 7
Clec4a2	C-type lectin domain family 4,member a2
cM	Centi Morgan
CNS	Central nervous system
CP	Chronic pancreatitis
CT	Computerized tomography
CTLA-4	Cytotoxic T-lymphocyte antigen 4
CTRC	Chymotrypsinogen C
Cypr5	Cytokine production 5 QTL
Dbm1	Diabetes modifier 1 QTL
DCIR	Dendritic cell immunoreceptor
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
E. Coli	Escherichia coli
Eae21	Experimental allergic encephalomyelitis susceptibility 21
Eae26	Experimental allergic encephalomyelitis susceptibility 26
Eae33	Experimental allergic encephalomyelitis susceptibility 33
EBA	Epidermolysis bullosa acquisita
ELISA	Enzyme-linked immunosorbent assay
F	Filial generation
F2	F2 intercross
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
Fig.	Figure
FITC	Fluorescein isothiocyanate *conjugated*
FoxP3	Forkhead box p3
FWB	FACS Wash Buffer

g	Gram
G1	First generation
G4	Fourth generation
GBS	Guillain-Barré syndrome
GEL	Granulocytic epithelial lesion
GN	Glomerulonephritis
H&E	Haematoxylin and eosin
HLA	Human leukocyte antigen,
hnRNPs	Heterogeneous nuclear ribonucleoproteins
HPF	High power field
HS	Heterogeneous stock
IBD	Inflammatory bowel disease
Ibdq2	Inflammatory bowel disease QTL 2
IC Ag	Intracellular antigens
ICDC	International consensus diagnostic criteria
IDCP	Idiopathic duct-centric pancreatitis
Idd27	Insulin dependent diabetes susceptibility
IDDM	Insulin dependent diabetes mellitus
INF-k	Interferon kappa
Ifnk	Interferon kappa gene
IFN-γ	Interferon gamma
IFN-γ R	Interferon gamma receptor
Ig	Immunoglobulin
IgG4	Immunoglobulin G4
IgG4 RsD	IgG4-related sclerosing disease
IgG4-RD	IgG4-related disease
IgG4-RSD	IgG4- related systemic disease
Igsf6	Immunoglobulin super family
IL10	Interleukin 10
Il12rb2	Interleukin 12 receptor, beta 2
Il17b	Interleukin 17b
Il17ra	Interleukin 17 receptor A gene
Il18bp	Interleukin 18 binding protein

Il1f10	Interleukin 1 family, member 10
Il1f5	Interleukin 1 family, member 5
Il1f6	Interleukin 1 family, member 6,
Il1f8	Interleukin 1 family, member 8
Il1f9	Interleukin 1 family, member 9
Il1rn	Interleukin 1 receptor antagonist
IL2	Interleukin 2
Il23r	Interleukin 23 receptor
Il2ra	Interleukin 2 receptor, alpha chain
IL4	Interleukin 4
Il4ppq	IL4 producing potential QTL
Il4ra	Interleukin 4 receptor, alpha gene
IL7	Interleukin 7
Irak2	Interleukin-1 receptor- associated kinase 2
ITP	Idiopathic Thrombocytopenic Purpura
IVIg	Intravenous immunoglobulin
IVIg	Intravenous immunoglobulin
Lat	Linker for activation of T cells
LF	Lactoferrin
LOD	Logarithm of the odds
Log p	Logarithm of P value
LP-BM5	Murine leukemia virus
LPSP	Lymphoplasmacytic sclerosing pancreatitis
LT	Lymphotoxin
Ltbr	lymphotoxin B receptor gene
Lxw1	Lupus BXSB x NZW 1
m	milli (10^{-3})
M	Molar (mol/dm^3)
mAb	Monoclonal antibody
MAX	Maximum
Mbp	Million base pairs/mega base pairs
mg/ml	Milligrams per milliliter
MHC	Major histocompatibility complex

min	Minute
mM	Millimolar
MRI	Magnetic Resonance Imaging
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
mtDNA	Mitochondrial DNA
n	Nano (10^{-9})
N2	N2 backcross generation
Nbwa2	NZB and NZW autoimmunity 2 QTL
NIH	National Institutes of Health
Nrf2	Nuclear factor, erythroid derived 2, like 2 gene
nTx	neonatal thymectomy
P	P value(s)
PAIL	Partial advanced Intercross lines
PBS	Phosphate buffered saline
PE	Phycoerythrin
PerCP-Cy5.5	Peridinin Chlorophyll protein -Cy5.5
PGIA	Proteoglycan-induced arthritis
Pgia27	Proteoglycan induced arthritis 27
poly I:C	Polyinosinic,polycytidylic acid
PPARγ	Peroxisome proliferator-activated receptor γ
PRSS1	Protease serine 1
PRSS2	Protease serine 2
Psds1	Psoriasis-like skin disease severity 1
PSTI	Pancreatic secretory trypsin inhibitor
Ptf1a	Pancreas specific transcription factor, 1a
QT	Quantitative trait
QTG	Quantitative trait gene
QTL	Quantitative trait loci
RA	Rheumatoid arthritis
RF	Rheumatoid factor
RIL	Recombination inbred lines
RNA	Ribonucleic acid

rpm	Rounds per minute
Sec.	Second
SjS	Sjogren syndrome
SLE	Systemic Lupus Erythematosus
Sle2	Systemic lupus erythmatosus susceptibility 2
SNP	Single nucleotide polymorphism
SPINK1	Serine protease inhibitor Kazal type1
Ssb	Sjogren syndrome antigen B
TCR	T cell receptor
Th1	T helper 1
Th17	Interleukin 17
Th2	T helper 2
Thcr	T helper cell response QTL
Tlf	T lymphocyte fraction
Tregs	Regulatory T cells
UBR2	Ubiquitin Protein Ligase E3 Component N-Recognin 2
Ubr3	Ubiquitin protein ligase E3 component n-recognin 3
μ	Micro (10^{-6})
μl	Microliter

9.2 List of Figures

9.3 List of Tables

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11 Curriculum Vitae

12 Statements

Here, I declare that I have written this thesis by myself with the only help of the quoted literature. I declare as well that this thesis has not been submitted to any other Institution before.

July 2014, Rostock