Autoimmune pancreatitis in MRL/Mp mice is a T cell-mediated disease responsive to cyclosporine A and rapamycin treatment

Theresa Schwaiger,1 Cindy van den Brandt,1 Brit Fitzner,2 Sarah Zaatreh,2 Franziska Kraatz,1 Annegret Dummer,1 Horst Nizze,3 Matthias Evert,4 Barbara M Bröker,5 Monika C Brunner-Weinzierl,6 Thomas Wartmann,7 Tareq Salem,3 Markus M Lerch,1 Robert Jaster,2 Julia Mayerle1

ABSTRACT

Background Autoimmune pancreatitis (AIP) in humans invariably responds to steroid treatment, but little is known about the underlying pathogenesis and the benefits of alternative treatments.

Objective To study the pathogenesis, and the efficacy of alternative immunosuppressant agents in the MRL/Mp mouse model of AIP.

Design MRL/Mp mice were pretreated for 4 weeks with polyinosinic:polycytidylic acid to induce AIP. Pancreatic sections of mice genetically deleted for CTLA-4 were analysed. Blockage of CTLA-4 was achieved by intraperitoneal antibody treatment with 2 μg/g anti-mouse-CD152. Subsequent therapeutic studies were performed for a period of 4 weeks using cyclosporine A (40 μg/g), rapamycin (1 μg/g) or azathioprine (15 μg/g).

Results Blockage of CTLA-4 in MRL/Mp mice suppressed regulatory T cell (Treg) function and raised the effector T cell (Teff) response with subsequent histomorphological organ destruction, indicating that AIP is a T cell-driven disease. Using an established histopathological score, we found that dexamethasone, cyclosporine A and rapamycin, but less so azathioprine, reduced pancreatic damage. However, the beneficial effects of cyclosporine A and rapamycin were achieved via different mechanisms: cyclosporine A inhibited Teff activation and proliferation whereas rapamycin led to selective expansion of Tregs which subsequently suppressed the Teff response.

Conclusions The calcineurin inhibitor cyclosporine A and the mammalian target of rapamycin (mTOR) inhibitor, rapamycin, improve the course of AIP in MRL/Mp mice via different mechanisms. These findings further support the concept of autoreactive T cells as key players in the pathogenesis of AIP and suggest that cyclosporine A and rapamycin should be considered for treatment of AIP in humans.

INTRODUCTION

Autoimmune pancreatitis (AIP) in humans is a rare cause of chronic pancreatitis (CP), part of a multorgan fibro-inflammatory syndrome, and significantly more common in Japan and Korea than among Caucasians. AIP is characterised by two histopathological patterns called lymphoplasmacytic sclerosing pancreatitis (type 1) and idiopathic duct centric pancreatitis (type 2), also known as granulocyte epithelial lesion-positive pancreatitis. Although the two entities are similar, the pathogenesis differs. 

Significance of this study

What is already known about this subject?

► Autoimmune pancreatitis (AIP) is a rare form of chronic pancreatitis characterised by peri-ductal infiltrates of inflammatory cells and, in severe cases, progressive destruction of acinar tissue.

► The presence of activated T lymphocytes in the pancreatic tissue and various serum autoantibodies (mostly against acinar cell-derived antigens) of patients with AIP suggests that the autoimmune response involves both a cellular and a humoral component.

► Although AIP can be successfully treated with steroids in most cases, little is known about the effectiveness of alternative treatments and their potential for maintaining remission and preventing relapse.

What are the new findings?

► Using the MRL/Mp mouse model of AIP as well as CTLA-4 deletion or inhibition we found that suppressing regulatory T cells increases the severity of AIP.

► Treatment that either expands the regulatory T cells or inhibits effector T cells has a beneficial effect comparable to steroid treatment.

► Of the three agents suitable for long-term treatment and prevention of relapse in humans—cyclosporine A, rapamycin and azathioprine—azathioprine was the least effective.

How might it impact on clinical practice in the foreseeable future?

► The MRL/Mp mouse model of AIP was shown to be suitable for investigating the underlying pathophysiology and also for testing treatment regimens for AIP.

► Experimental AIP is a T cell-driven disease, in which suppression of Tregs increases and inhibition of Teff cells reduces severity.

► Cyclosporine A and rapamycin are promising candidates for treatment of AIP in humans and should be tested in clinical trials, whereas azathioprine, in line with a recent case series by Hart et al13, appears less effective.

share common histopathological features (periductal lymphoplasmacytic infiltration and storiform periductal fibrosis), pathologists can distinguish them based on other unique histopathological features. Clinically, the two entities have a similar presentation (obstructive jaundice/pancreatic mass and a dramatic response to steroids) but differ significantly in their demography, serological characteristics, other organ involvement and disease relapse. Lymphoplasmacytic sclerosing pancreatitis is associated with raised titres of non-specific autoantibodies and serum IgG4, whereas idiopathic duct centric pancreatitis does not have definitive serological autoimmune markers.2–7

AIP and IgG4-related syndrome usually respond to steroids, with symptoms resolving within days or weeks, biochemical abnormalities becoming normal and radiographic evidence of inflammation disappearing. Although spontaneous remission can occur, the use of steroids has been shown to induce remission consistently and more rapidly than no treatment.8 Despite the high initial response rates to steroids, the disease will relapse in 15–60% of patients either on follow-up after stopping steroid treatment or during the initial steroid taper.8–11 Recent studies observed a morphological and clinical response to steroids in all their patients, but in almost 50% exocrine pancreatic insufficiency did not improve. This points towards treatment-refractory destruction of the gland, suggesting that a different approach to treatment may be needed.12 Unfortunately, besides steroids, little is known about the efficacy of other immunosuppressant agents apart from rituximab for the treatment of AIP in humans and randomised trials have not been carried out.13–17

Part of the reluctance to test alternative treatments stems from an incomplete understanding of the underlying pathophysiology. No disease-specific antibodies have been identified, whereas the reports of several disease-associated antibodies, such as anti-lactoferrin, anti-carbonic anhydrase II and IV, anti-pancreatic-secretory-trypsin inhibitor, anti-amylase α, anti-heat shock protein 10 and anti-plasminogen-binding protein peptide autoantibodies, in a simplistic world would suggest a Th2-triggered disease. On the other hand, the pronounced increase of IgG4 serum levels in patients with type 1 AIP indicates an imbalance between the effector T cell (Teff) and regulatory T cell (Treg) response. As a consequence, the disease progresses.18–20

CTLA-4, a surface protein of the Ig superfamily, is one of the most potent attenuators of T cell responses. CTLA-4 binds to B7.1 (CD80) and B7.2 (CD86) on antigen-presenting cells, interfering with the CD28-mediated signal required for priming naïve T cells, and the molecule is a prerequisite for Tregs to exert their suppressive function or inhibit the activity of Teff cells.21–26 Therefore, CTLA-4 has a dual role in maintaining self-tolerance, and blocking the interaction of CTLA-4 of Tregs and Teff with CD80 on antigen-presenting cells will enhance the immune response. Previously, a polymorphism of CTLA-4 has been reported to be associated with the disease phenotype of AIP suggesting an involvement in its aetiology.27–28 How CTLA-4 is involved in the pathogenesis of AIP and whether the CTLA-4/CD80 axis can be exploited for treatment is unknown.

To investigate how immunosuppression of T cells by CTLA-4 or specific immunosuppressive drugs affects AIP, we used a mouse model of AIP that mimics many histological features of human type 1 AIP such as storiform fibrosis, infiltrating IgG-positive plasma cells and obliterator phlebitis.2 29–36 A comparison of the phenotypic features of human AIP type 1 and AIP in MRL/Mp mice is given in table 1. Although spontaneous AIP of MRL/Mp mice is usually mild, application of polynosinic:polycytidylic acid (poly I:C) significantly accelerates and enhances the disease progression.30 33 To examine the role of CTLA-4 in AIP progression a CTLA-4 knockout model37 and a monospecific blocking antibody against CTLA-4 were employed. Based on the findings, we tested the therapeutic efficiency in AIP of three different immunosuppressant agents—the mammalian target of rapamycin (mTOR) antagonist, rapamycin; the anti-metabolite, azathioprine and the calcineurin inhibitor, cyclosporine A. Our results suggest that autoreactive T cells are key players in the pathogenesis of AIP and that cyclosporine A and rapamycin represent promising treatment alternatives to steroids in AIP.

### MATERIALS AND METHODS

#### Animals

MRL/Mp mice were purchased from Charles River Laboratory (Boston, USA).29–35 Female MRL/Mp mice at an age of about 30 weeks spontaneously develop CP with autoimmune characteristics. The key histopathological findings in diseased MRL/ Mp mice are inflammatory cell infiltrates that are mainly composed of CD4 or CD8 T cells. The lymphocytes are predominantly located around the interlobular ducts.2 Although spontaneous AIP in MRL/Mp mice is usually mild, application of poly I:C or recombinant interferon γ significantly accelerates and enhances disease progression.30 33 Female mice were used throughout the study as male MRL/Mp mice do not develop severe spontaneous autoimmune phenomena. Studies were started when the animals had reached an age of 3–4 months. Mice were housed in accordance with institutional and National Institutes of Health guidelines with a 12 h light–dark cycle. They had access to standard laboratory chow and water ad libitum. All experiments were performed according to the guidelines of the local animal use and care committee. Animals

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Phenotypic comparison between human type 1 AIP and AIP in MRL/Mp mice</th>
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<tbody>
<tr>
<td>Diagnostic criteria</td>
<td>Human type 1 AIP</td>
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<tr>
<td>Histological changes of the pancreas:</td>
<td></td>
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<tr>
<td>Periductal lymphoplasmacytic infiltration</td>
<td>+</td>
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<tr>
<td>Inflammatory infiltrate</td>
<td>+</td>
</tr>
<tr>
<td>Storiform fibrosis</td>
<td>+</td>
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<tr>
<td>Obliterator phlebitis</td>
<td>+</td>
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<tr>
<td>Plasma cells</td>
<td>+</td>
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<tr>
<td>IgG4 plasma cells</td>
<td>+</td>
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<tr>
<td>IgG4 serum levels (%)</td>
<td>50% (non-Asian)</td>
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<td>Anti-carboxyhydrazide antibodies (%)</td>
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<tr>
<td>Response to steroids</td>
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<tr>
<td>Other organ involvement (%)</td>
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<td>Pancreatitis</td>
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<tr>
<td>Inflammatory bowel disease</td>
<td>0.16</td>
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<tr>
<td>Intestinal pneumonia</td>
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AIP, autoimmune pancreatic; PSTI, pancreatic-secretory-trypsin-inhibitor. *Present but frequency not determined.
with genetic deletion of CTLA-4 have been previously described and were kindly provided by Dr Brunner-Weinzierl.\(^{37}\)

**Treatment protocol for the induction of AIP in MRL/Mp mice**

Female mice were randomised into the following groups (n=7 in each): poly I:C (5 μg/g body weight; Sigma-Aldrich, Deisenhofen, Germany) plus rapamycin (1 mg/g; Rapamune, Wyeth Pharma, Münster, Germany), cyclosporine A (40 mg/g; Sandimmun, Novartis Pharma, Nürnberg, Germany), azathioprine (15 μg/g Imurek; GlaxoSmithKline, Munich, Germany) or blocking anti-mouse CTLA-4 monoclonal antibody (1 mg/g; 9H10) and isotype antibody (both BD Bioscience, Heidelberg, Germany) as negative control. The control group received NaCl 0.9% instead of the medication. The precise treatment protocol is shown in figures 2 and 6. The doses of the immunosuppressant agents were chosen based on previous studies on autoimmune diseases in mice.\(^{38} - 44\) Poly I:C was injected intraperitoneally every third day according to the method previously described.\(^{32}\) Details of a pilot therapeutic study with dexamethasone (obtained from Sigma-Aldrich, Deisenhofen, Germany) are given in the legend to figure 1. Immunosuppressant/antibody treatment started 4 weeks after the first poly I:C injection. All substances were injected intraperitoneally (rapamycin and cyclosporine A daily, azathioprine three times a week and antibodies every third day).

At the indicated time point, mice were anaesthetised and peripheral blood collected in heparin-coated tubes. Spleen, liver, lung and pancreas were harvested and analysed by flow cytometry, fixed and cryo- or paraffin-embedded and used for further immunohistochemistry.\(^{29} 30\, 34\, 35\)

**Flow cytometric analysis of T cells**

To analyse subtypes of T cells splenic single-cell suspensions from treated mice and controls were prepared. A total of 1×10^6 leucocytes were stained with directly conjugated antibodies detecting surface markers such as anti-mouse CD3 eFluor 660 (17A2, eBioscience, Frankfurt, Germany), anti-mouse CD4 (40 μg/g; Sandimmun, Novartis Pharma, Nürnberg, Germany), azathioprine (15 μg/g Imurek; GlaxoSmithKline, Munich, Germany) or blocking anti-mouse CTLA-4 monoclonal antibody (1 μg/g; 9H10) and isotype antibody (both BD Bioscience, Heidelberg, Germany) as negative control. The control group received NaCl 0.9% instead of the medication. The precise treatment protocol is shown in figures 2 and 6. The doses of the immunosuppressant agents were chosen based on previous studies on autoimmune diseases in mice.\(^{38} - 44\) Poly I:C was injected intraperitoneally every third day according to the method previously described.\(^{32}\) Details of a pilot therapeutic study with dexamethasone (obtained from Sigma-Aldrich, Deisenhofen, Germany) are given in the legend to figure 1. Immunosuppressant/antibody treatment started 4 weeks after the first poly I:C injection. All substances were injected intraperitoneally (rapamycin and cyclosporine A daily, azathioprine three times a week and antibodies every third day).

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Teff were determined by staining with anti-mouse CD4 FITC and anti-mouse CD69 PE (H1.2F3). Tregs were detected by staining with anti-mouse CD4 FITC, and intracellular staining for anti-mouse CTLA-4 (UC10-4F10-11, both BD Biosciences, Heidelberg, Germany) and anti-mouse/human FoxP3 APC
(3G3, Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer’s instructions, and cells were analysed using FlowJo software.

Serum analysis

Lipase activity was measured by photometric assay (Roche, Grenzach-Whylen, Germany) and standardised using purified enzymes (Sigma, Deisenhofen, Germany).

Levels of IgGs in serum were measured using a mouse immunoglobulin isotyping kit with a dilution of 1:250,000 serum in phosphate-buffered saline (PBS; Merck Millipore, Darmstadt, Germany).

For determination of antinuclear antibodies (ANAs) in serum an ELISA was performed with a dilution of 1:50,000 serum in PBS (SunRed Biotechnology, Shanghai, China). All assays were performed according to the manufacturer’s instructions.

Histology and immunohistochemistry/immunohistology

For histological investigations pancreata were fixed in 4% formaldehyde phosphate buffer overnight and processed for paraffin embedding. Routine haematoxylin and eosin (H&E) staining for determination of organ inflammation was performed on 1μm sections using standard procedures. Histopathological evaluation of pancreatic lesions was performed by light microscopy. The severity of inflammation was determined by scoring the degree of inflammatory cell infiltration into tissues as described by Kanno et al (0=none; 1=mild; 2=moderate; 3=moderate and diffuse or severe but focal; 4=severe and diffuse). The scores of three sections were averaged to give a final score. Scoring was done by at least two experienced independent reviewers and when there was disagreement by joint review with a third investigator (JM or RJ).

Masson–Goldner staining was used for visualisation of connective tissue on formalin-fixed sections, according to the manufacturer’s instructions (MerckMillipore, catalogue no 100485, Darmstadt, Germany).

Infiltrating inflammatory cells were further classified by immunohistochemical analysis. For staining of Tregs, air-dried cryostat sections of pancreas (2μm) were first fixed by incubation in ice-cold methanol for 20 min, air-dried again, incubated for 10 min with PBS containing 20% fetal calf serum (FCS) followed by 10 min exposure to 50μl 1.3% H2O2 for cell permeabilisation. Sections

Figure 3  Effect of CTLA-4 deletion or blockage on pancreatic lesions in MRL/Mp mice. Genetic deletion of CTLA-4 (A) resulted in spontaneous pancreatitis with a pronounced lymphoplasmic infiltrate, perivenular infiltration of lymphocytes progressing to phlebitis (arrow) and destruction of acinar tissue (*). Fibrosis is depicted by a bullet-ended arrow. MRL/Mp mice (B–F) were pretreated with poly I:C (n=7 per group) followed by application of a blocking CTLA-4 antibody or isotype antibody. (B) Haematoxylin and eosin staining of the isotype control with perivenular infiltration; in the centre a small sclerosing duct next to it. Application of the blocking antibody led to progressive infiltration of inflammatory cells, sclerosing ducts and destruction of acinar cell tissue architecture (C). The increase in severity is shown in (D), with mean severity scores±SEM for both groups. *p<0.05 versus isotype control mice. Representative results for (E) isotype control antibody and (F) anti-CTLA-4 antibody showing an overall increase in fibrosis detected by Goldner–Masson staining.

were washed twice with PBS, and incubated for 10 min with an avidin D solution, followed by a 15-min treatment with a biotin solution to block endogenous biotin (Avidin/Biotin blocking kit, DAKO Cytomation, Glostrup, Denmark), washed again and subsequently incubated with hamster anti-mouse CTLA-4 overnight at 4°C. After incubation with biotinylated anti-hamster IgG slides were washed and the signal was enhanced by using a TSA system (Perkin Elmer, Massachusetts, USA). Next, slides were treated with streptavidin-TRITC (Jackson ImmunoResearch, Pennsylvania, USA), anti-mouse FoxP3-Alexa 488 and anti-mouse CD4-Alexa 647 (both eBioscience, Frankfurt, Germany) for 1 h in the dark. Finally, slides were washed and analysed by fluorescence microscopy.

For staining of macrophages, acetone-fixed cryosections of pancreatic tissue were first blocked with 20% FCS in PBS, followed by incubation overnight with anti-mouse CD68 (mouse anti-mouse) and anti-mouse CD163 (rat anti-mouse) antibodies (both http://www.antikuerper-online.de). After two washing steps, slides were treated with the secondary antibodies anti-mouse Cy-3 IgG (Jackson ImmunoResearch, West Grove, Pennsylvania, USA) and anti-rat FITC IgG (Invitrogen, Darmstadt, Germany) for 1 h in the dark, followed by counterstaining with 4',6-diamidino-2-phenylindole. Slides were washed and analysed by fluorescence microscopy.

Staining of B cells and T cell subsets was performed as follows: after 1 h of blocking with 20% FCS in PBS, slides were incubated for 1 h in the dark with the directly conjugated antibodies anti-mouse IgG DyLight 488, anti-mouse CD138 PE and anti-mouse CD19 Alexa Fluor 647 for plasma cells and B cells or anti-mouse CD4 FITC, anti-mouse CD8a PE and anti-mouse CD69 Alexa Fluor 647 for T cell subsets (all antibodies, BioLegend, Fell, Germany). Finally, slides were washed and analysed by fluorescence microscopy.

Morphometric quantification was performed on five randomly taken pictures for each animal in seven animals at a magnification of ×400 and positive cells were counted manually. Mean values ±SD are given in the figures.

Statistical analysis
The data are presented as the mean±SEM unless otherwise stated. All statistics were calculated using GraphPad PRISM (V5.0; GraphPad, San Diego, California, USA). Statistical evaluations were performed by using non-parametrical tests (Mann–Whitney). p Values of <0.05 were considered statistically significant and n represents the number of animals in the figure legends.

RESULTS
Dexamethasone is an efficient treatment for AIP in MRL/Mp mice
In agreement with a previous study by Qu et al.,13 MRL/Mp mice treated with poly I:C developed severe histopathological changes of the pancreas corresponding to AIP with a mean severity of >2, including storiform fibrosis, obliterator phlebitis and IgG-positive plasma cells (figure 1A, column 2, see online supplementary figure S4C), while the mean score of untreated age-matched MRL/Mp mice was <1 (figure 1A, column 1). Although older MRL/Mp mice also developed signs of spontaneous AIP29–32 we chose for our subsequent studies the use of poly I:C to achieve a more consistent and reproducible disease severity. To confirm that the model was suitable for a therapeutical study, we initially tested the efficacy of dexamethasone. During the last 2 weeks of a 6-week course of poly I:C treatment mice received daily injections of up to 1 μg/g of dexamethasone. As shown in figure 1A,B, dexamethasone reduced the pathological changes of the pancreas in a dose-dependent manner. At the highest dose of 1 μg/g, the mean score was <1 and the H&E staining was similar to that of controls. Figure 1B shows typical pancreatic lesions in the group treated with poly I:C alone (destruction of exocrine tissue, large infiltrates of inflammatory cells and fibrosis) and the effect of dexamethasone at 1 μg/g (largely preserved exocrine tissue; few inflammatory cells). For other organ involvement in this model such as kidney and liver as a hallmark of AIP type 1 see online supplementary figure S1. These changes were detectable, could be influenced by treatment, but in general were mild.

Blockade of CTLA-4 results in an increased severity of AIP in MRL/Mp mice
The importance of the B7–CD28/CTLA-4 system in vivo has been illustrated in studies that blocked this pathway to exacerbate general autoimmune in rodents.33,34 Of note, we found that CTLA-4 knockout animals at the age of 21 days develop spontaneous pancreatitis with a prominent lymphoplasmic infiltrate, a CD4-positive infiltrate around sclerosing ducts, perivenular lymphocyte infiltration progressing to obliterator phlebitis (figure 3A), increased numbers of activated T cells (see online supplementary figure S2A), CD4/CD25/FoxP3 cells (see online supplementary figure S2B), B cells and IgG-positive plasma cells (see online supplementary figure S2C) as well as infiltration of M1 and M2 macrophages (see online supplementary figure S2D) and destruction of acinar tissue compared with healthy littersmates (figure 3A, insert). CTLA-4-deficient animals succumb to their immunoproliferative syndrome within 25 days after birth.

The morphological changes seen in the CTLA-4 knockout animals paralleled the severe form of AIP in MRL/Mp mice and prompted us to study the effect of a CTLA-4 blocking antibody in MRL/Mp mice treated with poly I:C for 4 weeks. As expected, MRL/Mp mice treated with poly I:C for up to 54 days displayed severe histopathological changes of the pancreas (figures 1A,B and 3B,D). Treatment with a monoclonal anti-mouse CTLA-4 antibody starting 4 weeks after the first poly I:C injection and continued for 24 days (for details see figure 2) increased the histopathological severity score significantly from an average of 2.0±0.31 to 2.85±0.14 (p=0.037). CTLA-4 blockade resulted in a pronounced inflammatory cell infiltration with destruction of pancreatic ducts, a perivenular infiltration and acinar cell deletion (figure 3C). On Goldner staining (figure 3F) extracellular collagen deposits around ducts and venular structures increased in animals treated with the anti-CTLA-4 antibody (figure 3F) compared with isotype antibody-treated controls (figure 3E).
CD69 (activated Teff), FoxP3 (Treg) and CD25 on CD4 cells. We observed a shift between T cell subsets, resulting in a decrease of naïve T cells (CD62L-positive cells figure 4E) in conjunction with an increase of activated T eff cells (CD69 cells figure 4F). While the rate of resting T cells decreased (figure 4E) upon CTLA-4 blockade, the rate of Teff (figure 4F) increased as did the rate of Tregs (figure 4G). Even though the subset of Tregs (FoxP3 CD4 cells, figure 4G) significantly increased, this population is probably non-suppressive owing to the blockade of CTLA-4, because otherwise the increased number of Tregs would have prevented or at least dampened the massive activation of T effs (figure 4F, figure 9). In line with this we did not detect an increase in the mean fluorescence intensity for the activation marker CD25 on Tregs, which would be mandatory for the suppressive capacity of Tregs (figure 4H).

The systemic response to the blockade of CTLA-4 did not explain the increase in local severity of AIP in the pancreas. Serum lipase levels pointed to an increased local damage in mice receiving the anti-CTLA-4 antibody (see online supplementary figure S3A). To characterise the composition of inflammatory cells at the inflamed side of the pancreas, pancreatic tissue was analysed for CD68, CD163, CTLA-4 and FoxP3 on CD4 cells. A quantification of the macrophage M1/M2 population showed a predominance of M1 macrophages over M2 macrophages (figure 5A,B). A comparison of the CTLA-4 antibody-treated group and the isotype control showed a three-fold induction of CD4 cells upon CTLA-4 blockade but a decrease in Tregs (figure 5C–F). We further found a predominance of CD4 cells over CD8 cells infiltrating the pancreas, scarcely any B cells (CD19), but an increase in IgG-positive plasma cells in the pancreas of CTLA-4-Ab-treated mice (see online supplementary figure S4A–D). Studying IgG subclasses we found no difference between the two groups and were therefore unable to identify the equivalent of IgG4 in humans (see online supplementary figure S5A–E). This suggests that tissue destruction is mediated by activated Teff cells and treatment should aim directly or indirectly at silencing a Teff response.

Treatment with cyclosporine A and rapamycin but not with azathioprine reduces the severity of AIP
To study the efficacy of three different immunosuppressant agents, cyclosporine A, rapamycin and azathioprine, for the treatment of AIP we pretreated animals for 4 weeks with poly I:C and started to inject immunosuppressant agents at day 28 for another 4 weeks (figure 6). NaCl controls had, on average, a mean severity score of 2.4±0.2 (figure 7A,E), whereas treatment with either cyclosporine A or rapamycin significantly reduced severity to 1.7±0.18 and 1.4±0.2, respectively (figure 7B,C,E). Surprisingly, azathioprine did not reduce the severity of AIP (figure 7D,E). Moreover, one mouse of the azathioprine group

Figure 4 Effect of CTLA-4 blockage on the different T cell populations isolated from the spleen. MRL/Mp mice were pretreated with poly I:C (n=7 per group) followed by application of a blocking CTLA-4 antibody or isotype antibody. Fluorescence-activated cell sorting analysis of splenic T cells ruled out a change in overall CD3 cells upon antibody blockage of CTLA-4 (A). CTLA-4 blockage resulted in a decrease in CD8 T cells (B), with a simultaneous increase in CD4 T cells (C), no change in the overall effector T cell population (D), but a reduction in naïve T cells (E) and a pronounced increase in activated T cells (F). The number of Tregs increased (G), whereas the activity of Tregs remained unchanged upon antibody treatment (H). Indicated are percentages as means±SEM for both groups. *p<0.05 versus isotype control mice. MFI, mean fluorescence intensity.

died on day 26 of treatment and could not be included in the further analysis. H&E staining of cyclosporine A and rapamycin-treated animals showed a less prominent inflammatory infiltrate, preserved tissue architecture, decreased obliterative phlebitis and less fibrosis (figure 7A–D). Goldner–Masson staining disclosed decreased fibrosis in the rapamycin-treated group (figure 7F, insert figure 7G NaCl). Taken together, these results demonstrate that immunosuppressive drugs which suppress T cell responses are effective for treatment of experimental AIP.

Figure 5  Effect of CTLA-4 blockage on the infiltration of leucocytes into the pancreas. MRL/Mp mice were pretreated with poly I:C (n=7 per group) followed by application of a blocking CTLA-4 antibody or isotype antibody. Cryosections from pancreatic tissue either treated with an isotype control antibody (A) or an anti-CTLA-4 blocking antibody (insert) were co-labelled against CD68 (red) and CD163 (green) and counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue). Fluorescence staining showed many M2 macrophages (stained red and green) in control mice. Quantification of M1 and M2 macrophages indicated a nearly 1:1 distribution of M1 to M2 macrophages in control mice, whereas the anti-CTLA-4-Ab-treated mice showed a shift towards M1 macrophages (B). Co-staining of CD4 (blue), CTLA-4 (red) and FoxP3 (green) in cryosections of pancreatic tissue showed a prominent increase in CD4 cells and a decrease in triple positive cells (Treg) from mice treated with the a-CTLA-4-Ab (D) in comparison with control mice receiving isotype antibody (C). Indicated is the number of CD4 cells per field (means±SEM) for both groups (E) and the percentage of triple positive cells of CD4 cells (F) (means±SEM). *p<0.05 versus isotype control mice.

Figure 6  Treatment schedule with cyclosporine A, rapamycin and azathioprine in the MRL/Mp mouse model of autoimmune pancreatitis. For details, please refer to the ‘Materials and methods’ section.
Characterisation of local and systemic T cell response in AIP after immunosuppressive treatment

Of the three immunosuppressive agents tested, only cyclosporine A reduced splenic CD3 T cells (p<0.05) (figure 8A). Rapamycin, on the other hand, increased the number of CD8 cells and reduced CD4 cells, suggesting two different mechanisms of immunosuppressive action (figure 8B,C). Azathioprine did not quantitatively affect the T cell population. With respect to the number of T effs all three agents reduced the number of splenic FoxP3−CD4 T cells (figure 8D). While rapamycin and azathioprine expanded the T reg population (CTLA-4/FoxP3/CD4 cells, figure 8G), cyclosporine A acted via a decrease in T eff population (CD69/CD4 cells, figure 8F). The effect of azathioprine in expanding the T reg population in the absence of an increase in CD25 mean fluorescence intensity on FoxP3 cells (figure 8H), and in combination with an overall decrease in CD62L expression on CD4 cells (figure 8E), indicates a non-suppressing Treg population. Ultimately, the effect of azathioprine on Tregs was not sufficiently sustained to affect the T eff population responsible for tissue destruction in the pancreas. This explains the lack of a treatment effect of azathioprine on experimental AIP (figure 7D,E).

A study of the composition of macrophages invading the pancreas showed a significant decrease for the proinflammatory M1 macrophage population upon rapamycin treatment (figure 9A–C). The immunofluorescence for CD4/FoxP3/CTLA-4 indicated a prominent T cell infiltrate in the pancreas in untreated animals, which was similar in azathioprine-treated mice (figure 9D,G). Importantly, the prominent T cell infiltrate was almost absent in cyclosporine A (figure 9E) and rapamycin (figure 9F)-treated animals. We neither detected a change in the splenic B cell population nor did we find a meaningful shift of IgG subclasses or ANA titres before or after treatment (see online supplementary figures S6–8). However, the IgG-positive plasma cell rate was markedly decreased in the cyclosporine and rapamycin group while rapamycin even reduced the number of B cells in the pancreas (see online supplementary figure S6 and S7A).

DISCUSSION

The molecular pathogenesis of human AIP remains largely unknown. In some patients with AIP, circulatory Th1 cells predominate over Th2 type cells.18,48 On the other hand, a Th2-type immune reaction is induced in the liver during IgG4-related sclerosing cholangitis together with a Th1 response.49 The discrepancy...
may be explained by a shift of Th2 cells from the periphery to local tissues, or is probably, due to different disease stages. Recent studies have suggested a biphasic disease mechanism of induction and progression. The initial response to self-antigens, a number of which have been reported (eg, lactoferrin, carboanhydrase II, carboanhydrase IV, pancreatic-secretory-trypsin inhibitor, α-amylase, PBP peptide of Helicobacter pylori, trypsinogen and n-recognin), might be induced by a decrease in naïve T regs and then followed by a Th2 response. In the progression phase T regs are upregulated in patients with AIP and those cells constitutively express CTLA-4. The suppressive function of T regs is mediated via cell-to-cell contact ligation of CTLA-4. In line with this observation our investigations of pancreatic sections from CTLA-4 knockout animals showed an identical pattern of tissue destruction to that seen in MRL/Mp mice stimulated with poly I:C. Genetic studies of patients with AIP have reported an association with a loss-of-function polymorphism in the CTLA-4 gene, suggesting a regulatory role for this molecule in AIP. This regulation could involve the balance between T effs, on which CTLA-4 is expressed upon activation to control for overwhelming activation, and T regs in AIP. An imbalance between the two might cause tissue destruction upon Teff cell activation.

To further clarify the morphological similarity with the CTLA-4 knockout model and to examine the pathophysiological role of CTLA-4 we employed a CTLA-4 blocking antibody in the MRL/Mp model of AIP. In line with our hypothesis we detected an increased severity of AIP after blockage of CTLA-4. This was mediated by an increase in splenic T eff cells as well as overall splenic T regs. This parallels the human situation. Our experiments suggest that the decrease in naïve T regs and the increase in T eff cell activation is a pathogenetic factor for the disease and makes it attractive as a therapeutic target.

Although several studies have dealt with different aspects of the MRL/Mp model and its resemblance to type 1 AIP, the efficacy of immunosuppressant treatment has never been studied.

Azathioprine is one of the best established immunosuppressant agents in autoimmune disease. Its mode of action interferes with replication of DNA in lymphocytes, which lack a salvage pathway for purine synthesis, and also with the CD28 costimulation of alloreactive T lymphocytes. The latter convert the costimulatory signal from CD28 to an apoptotic signal. Azathioprine thereby deletes activated lymphocytes and has been regarded as an ideal treatment for AIP. As azathioprine is the best studied immunosuppressant agent in humans and has also been used in human AIP we investigated this agent in our animal model. To our surprise...
azathioprine used for the induction of remission did not have a markedly beneficial effect on AIP besides a slight decrease in T eff and an increase in T reg at therapeutic concentrations previously used for the treatment of autoimmune hepatitis. Although this finding was unexpected, it is in line with the recent report by Hart et al who could not show that azathioprine prevented a relapse of AIP in humans.

We tested a second therapeutic agent known to selectively act on the T cell response—the calcineurin inhibitor cyclosporine A. The mechanism of action for cyclosporine A is to form an intracellular complex with immunophilins (eg, cyclophilin) which inhibits calcineurin activity and thus prevents nuclear translocation of N-FAT and cytokine gene transcription. The net result is that cyclosporine A blocks the production of cytokines such as interleukin (IL)-2 and inhibits T cell activation and proliferation. However, even though calcineurin inhibitors have shown some positive effect on T cell apoptosis in the aly/aly mouse model of AIE cyclosporine A did not achieve a beneficial effect in comparison with rapamycin, the third substance we investigated in our model.

There are a number of reasons why rapamycin may be more effective. The mTOR antagonist rapamycin prevents progression of effector T cells from the G0 to G1 phase of the cell cycle by stopping translation of IL-2 and the IL-2R gene, thus decreasing the rate of T cell proliferation. The autocrine IL-2 loop is mandatory for progression through the first checkpoint of the cell cycle. However, Tregs can still proliferate in the presence of rapamycin because they do not activate the PI3K-AKT-mTOR
pathway upon T cell receptor binding, and, on the other hand, FoxP3 reinforces IL-2 secretion via STAT5.56 Furthermore, in non-obese diabetic mice and for CP in Bono/Kobori rats, mTOR inhibition suppresses the immune response and was found to be beneficial.37–39 Taken together, these observations suggest that rapamycin may be an ideal treatment for AIP.

In our animal model of experimental type 1 AIP we detected a highly significant treatment effect which surpassed the effect of cyclosporine A and was based in the pathophysiological pathway of rapamycin action as discussed above. The selective expansion of Tregs, paralleled by a decrease in activated T eff cells, was responsible for the observed treatment effect. Interestingly, none of the treatment regimens we employed showed a significant effect on B cell responses, and no decrease in one of the immunoglobulin subclasses or ANA level was found (see online supplementary figures S6–8). However, recent case reports have suggested that rituximab, a B cell-directed blocking antibody, might be beneficial as a rescue treatment in type 1 AIP.

In conclusion, in a mouse model which resembles human AIP type 1 in many aspects we could show that CTLA-4 and auto-reactive T cells are critically involved in the pathogenesis of the disease. Our data further suggest that blocking the mTOR pathway is an alternative treatment to steroids and maybe more suitable for long-term use. As mice are not men it should be investigated in humans with AIP.

Author affiliations
1 Department of Medicine A, University Medicine, Ernst-Moritz-Arndt University Greifswald, Greifswald, Germany
2 Department of Medicine II, Division of Gastroenterology, University of Medicine, University of Rostock, Rostock, Germany
3 Institute of Pathology, University Medicine, University of Rostock, Rostock, Germany
4 Institute of Pathology, University Medicine, Ernst-Moritz-Arndt University Greifswald, Greifswald, Germany
5 Division of Immunology, Institute of Immunology and Transfusion Medicine, University Medicine, Ernst-Moritz-Arndt University Greifswald, Greifswald, Germany
6 Department of Pediatrics, Division of Pediatric Immunology, Otto-von-Guericke University Magdeburg, Magdeburg, Germany
7 Division of Experimental Surgery, Otto-von-Guericke University Magdeburg, Magdeburg, Germany

Acknowledgements
We gratefully acknowledge the excellent technical assistance of Mrs Katja Bergmann, Mrs Kathrin Gladrow, Mrs Sarah Westerhold and Mrs Katrin Siewert-Küchenmeister. We acknowledge Professor Dr Jörg Emmrich, who sadly died in June 2011, for his intellectual input.

Contributors
TS, CvdB, S2, FK, AD: acquisition of the data, analysis and interpretation of the data and drafting of the manuscript. ME, SA, TN, HS, RJ: study concept and design, drafting of the manuscript, obtained funding and study supervision.

Funding
This work was supported by the Eva Luise and Horst Köhler Foundation, the Deutsche Forschungsgemeinschaft (DFG GRK40-2:DE3/E4, MA 4115/1-23, EV 168/2-1), the Federal Ministry of Education and Research (BMBF GANI-MED 03IS0261A and BMBF 03141070, 01ZZ9603, 01ZZ1003, 01ZZ2043, 03ZK012) and the European Union (EU-FP-7: EPC-TM and EU-FP7-REGPOT-2010-1).

Competing interests
None.

Provenance and peer review
Not commissioned; externally peer reviewed.

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Autoimmune pancreatitis in MRL/Mp mice is a T cell-mediated disease responsive to cyclosporine A and rapamycin treatment

Theresa Schwaiger, Cindy van den Brandt, Brit Fitzner, et al.

Gut 2014 63: 494-505 originally published online April 5, 2013
doi: 10.1136/gutjnl-2012-303635

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