Lymphotoxin receptor signaling promotes development of autoimmune pancreatitis

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Abstract: BACKGROUND AIMS:: Little is known about the pathogenic mechanisms of autoimmune pancreatitis (AIP), an increasingly recognized, immune-mediated form of chronic pancreatitis. Current treatment options are limited and disease relapse is frequent. We investigated factors that contribute to development of AIP and new therapeutic strategies. METHODS:: We used quantitative PCR, immunohistochemical and ELISA analyses to measure expression of cytokines and chemokines in tissue and serum samples from patients with and without AIP. We created a mouse model of human AIP by overexpressing LT and specifically in acinar cells (Ela1-LTab mice). RESULTS:: mRNA levels of lymphotoxin (LT) and were increased in pancreatic tissues from patients with AIP, compared with controls, and expression of chemokines (CXCL13, CCL19, CCL21, CCL1 and BAFF) was increased in pancreatic and serum samples from patients. Upregulation of these factors was not affected by corticosteroid treatment. Acinar-specific overexpression of LT (Ela1-LT) in mice led to an autoimmune disorder with various features of AIP. Chronic inflammation developed only in the pancreas but was sufficient to cause systemic autoimmunity. Acinar-specific overexpression of LT did not cause autoimmunity in mice without lymphocytes(Ela1-LT/Rag1(-/-)); moreover lack of pro-inflammatory monocytes (Ela1-LTab/Ccr2-/-) failed to prevent AIP but prevented early pancreatic tissue damage. Administration of corticosteroids reduced pancreatitis but did not affect production of autoantibodies, such as anti-pancreatic secretory trypsin inhibitor in Ela1-LTab mice. In contrast, inhibition of LT R signaling reduced chemokine expression, renal immune-complex deposition, and features of AIP in Ela1-LTab mice. CONCLUSIONS:: Overexpression of LT specifically in acinar cells of mice causes features of AIP. Reagents that neutralize LT R ligands might be used to treat patients with AIP.

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Lymphotoxin β Receptor Signaling Promotes Development of Autoimmune Pancreatitis

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Disclosure/Conflict of Interest

Stephan Segerer receives benefits from ROCHE as a consultant. Jeffrey Browning is an employee of Biogenidec and is involved with the development of LTBR-Ig.

The remaining authors declare no conflicts of interest.

Author contributions: GS designed and performed experiments, analyzed data and wrote the manuscript; TR, FR, YS, AM, SS, LS, SR, ML, DM, MB, ES, TR performed experiments; TK, YH, CL, JH, EA, AP provided human material and participated in paper writing; JB provided murine material and participated in paper writing, WA contributed to the pathological analysis of tissues and editing the manuscript, HA, AM, MM, MA, AA, MP participated in editing the manuscript and provided conceptual advice; RG and MH designed the study, analyzed the data and wrote the manuscript.

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Abbreviations used in the paper: AIP, autoimmune pancreatitis; ANA, anti-nuclear antibodies; CP, chronic pancreatitis; FDC, follicular dendritic cells; HEV, high endothelial venules; LT, Lymphotoxin; LF, lactoferrin; PSTI, pancreatic secretory trypsin inhibitor; TLT, tertiary lymphoid tissue; TNF, tumor necrosis factor; Treg, regulatory T-cells;
**Background & Aims:** Little is known about the pathogenic mechanisms of autoimmune pancreatitis (AIP), an increasingly recognized, immune-mediated form of chronic pancreatitis. Current treatment options are limited and disease relapse is frequent. We investigated factors that contribute to development of AIP and new therapeutic strategies.

**Methods:** We used quantitative PCR, immunohistochemical and ELISA analyses to measure expression of cytokines and chemokines in tissue and serum samples from patients with and without AIP. We created a mouse model of human AIP by overexpressing LTα and β specifically in acinar cells (Ela1-LTab mice).

**Results:** mRNA levels of lymphotoxin (LT) α and β were increased in pancreatic tissues from patients with AIP, compared with controls, and expression of chemokines (CXCL13, CCL19, CCL21, CCL1 and BAFF) was increased in pancreatic and serum samples from patients. Upregulation of these factors was not affected by corticosteroid treatment. Acinar-specific overexpression of LTαβ (Ela1-LTab) in mice led to an autoimmune disorder with various features of AIP. Chronic inflammation developed only in the pancreas but was sufficient to cause systemic autoimmunity. Acinar-specific overexpression of LTαβ did not cause autoimmunity in mice without lymphocytes (Ela1-LTab/Rag1-/-); moreover lack of pro-inflammatory monocytes (Ela1-LTab/Ccr2-/-) failed to prevent AIP but prevented early pancreatic tissue damage. Administration of corticosteroids reduced pancreatitis but did not affect production of autoantibodies, such as anti-pancreatic secretory trypsin inhibitor in Ela1-LTab mice. In contrast, inhibition of LTβR signaling reduced chemokine expression, renal immune-complex deposition, and features of AIP in Ela1-LTab mice.

**Conclusions:** Overexpression of LTαβ specifically in acinar cells of mice causes features of AIP. Reagents that neutralize LTβR ligands might be used to treat patients with AIP.

**Keywords:** Tertiary lymphoid tissues; immune regulation; Regulatory T cell, TNF superfamily
Introduction

Chronic pancreatitis (CP) is an inflammatory disease of the exocrine pancreas with irreversible tissue damage resulting in severe exocrine and endocrine insufficiency [1]. Chronic destruction of acinar cells usually coincides with inflammation, metaplasia and fibrosis. Autoimmune pancreatitis (AIP) is a distinct form of CP with unknown aetiology, but with characteristic clinical, histological features [2]. 11-15% of all CP cases presumably develop in an autoimmune context [3]. Two types of AIP have been described: (1) Type 1 AIP develops into a systemic IgG4-positive disease, with pancreatic lymphoplasmacytic inflammation, germinal center formation and vasculitis, (2) Fibro-inflammatory duct-centric type (type 2 AIP) with granulocyte epithelial lesions and pancreatic duct destruction without IgG4-positive cells or systemic involvement [4].

Elevated IgG4 levels are currently the most sensitive serum parameter for diagnosis [5]. However, about half of all AIP patients have normal serum IgG4 levels, and elevated IgG4 levels were reported in non-autoimmune pancreatitis and pancreatic cancer [6]. Recently, B-cell activating factor (BAFF) was identified as a novel serum marker for diagnosis and treatment response of AIP in combination with total serum IgG and IgG4 or anti-nuclear antibodies (ANA) [7].

Auto-antibodies have been detected in human AIP against carbonic anhydrase II (anti-CA-II), lactoferrin (anti-LF) and trypsinogen [8]. Current studies revealed that approximately 40% of AIP patients suffer from extra-pancreatic manifestations involving liver, salivary glands, retroperitoneum or kidneys [9]. Thus, AIP is a systemic autoimmune disease, responding to conventional steroid treatment [3]. However, relapses are frequent (~40%) and are associated with a more severe disease course [10]. Hence, novel therapeutic strategies and better understanding of the underlying disease mechanisms are needed.

Major regulators of immunity under normal and pathological conditions include the cytokines LTα, LTβ and their receptor (LTβR), members of the tumor necrosis factor (TNF) superfamily. Under physiological conditions, activated immune cells express LT. Under pathological conditions, parenchymal cells - like hepatocytes - are also capable of expressing
LT [11]. LTβR-signaling serves pleiotropic functions including development of lymphoid organs [12] and control of lipid homeostasis [13]. LT is crucial for generation and maintenance of tertiary lymphoid tissues (TLTs), frequently arising during chronic inflammation [11] [14] [15] and in many autoimmune diseases (e.g. rheumatoid arthritis, Sjögren’s syndrome) [16,17]. TLTs upregulate LT, express chemokines, adhesion molecules and contain cells or structures depending on LTβR-signaling (e.g. follicular dendritic cells (FDC), high endothelial venules (HEVs)) [14,18-21]. Ectopic LT expression was previously investigated in transgenic animal models such as RIP-LTα [22], AlbLTαβ [11] and IckLTαβ [23]. Although these models display TLTs in the respective organs, they lack autoimmune disease.

Human AIP tissue displayed strong up-regulation of LTαβ and target genes. Thus, we created transgenic mice expressing LT under the control of the acinar-cell specific elastase promoter (Tg(Ela1-LTa,b)) to test the potential role of LT in AIP pathology. We utilized Tg(Ela1-LTa,b) mice to investigate AIP disease mechanisms, and to explore novel therapeutic strategies for AIP.
Materials and Methods:

Human samples: Human pancreas biopsies and serum samples were obtained from the University Hospitals Zurich and Bern, the University of Graz and Ehime University. All samples were registered in the respective biobanks and kept anonymous. The research project was authorized by the Ethics Committee of the Canton of Zurich (Ref. Nr.StV 26-2005), local ethics committee Bern, Graz (Ref. Nr 20-492 ex 08/09), and the Ehime University Graduate School of Medicine (#1001003). The study followed the ethical guidelines of the Helsinki declaration.

Antinuclear antibody (ANA) detection: Sera obtained from Tg(ELa1-Lta,b) and C57BL/6 mice at the age of 6, 12 and 18 months were used to detect the presence of ANA using Hep2 cells (Euroimmun Lübeck, Germany). The cells were incubated with 1:60 and 1:200 dilutions of each mouse serum sample in PBS, followed by incubation with FITC-conjugated polyclonal goat antibody to mouse IgG-H&L (Abcam, Cambridge, UK). The reaction was visualized using a LEICA DLMB immunofluorescence microscope (absorption, 485–520 nm; emission, 520–560 nm wavelengths) Staining intensity was determined visually by two independent experienced technicians of the diagnostic immunology laboratory.

Statistical analyses and software: GraphpadPrism version 5 (LaJolla, Ca) was used to construct figures and diagrams. One-way ANOVA or unpaired t-tests and Mann-Whitney tests were used where appropriate. Differences were considered statistically significant if $P < 0.05$ and marked with an asterisk*: $P < 0.05$; **: $P < 0.01$, ***: $P < 0.001$.

Additional methods are described in the supporting information.
Results

Distinct expression patterns in non-AIP versus AIP patients

We first analysed samples derived from inflammation-unaffected patients, patients with pancreatic inflammation, and AIP-patients (Supplementary Table 1A) by qPCR for the expression of cytokines and chemokines. Identical tissue specimens used for mRNA expression were pre-screened by H&E staining verifying respective pancreatic pathologies (Figure 1A). Strong upregulation of \(LT\alpha\), \(LT\beta\), CXCL13, CCL19, CCL21, CCL17 and BAFF was found in AIP patients (Figure 1B, Supplementary Figure 1A). IL17, which was recently proposed as crucial for autoimmune disease and TLT development [24] in non-lymphoid organs was not significantly induced. In contrast, increase in CCL20, TNF\(\alpha\), CCL2, IL6, \(\alpha\)-SMA and IL17 transcripts was found in fibrotic, non-AIP pancreata compared to healthy controls or AIP patients (Figure 1B; Supplementary Figure 1B).

LT expression on infiltrating lymphocytes and metaplastic acinar cells

We next aimed at identifying cells expressing LT in pancreata of CP and AIP patients (Supplementary Table 1B). Immunohistochemistry of pancreatic tissues revealed strong LT\(\beta\) protein expression on infiltrating lymphocytes and acinar cells in non-AIP patients with fibrotic pancreatitis and AIP patients compared to control tissues. In most cases, the latter underwent acinar-ductal-metaplasia (ADM) (Figure 1C). No overt difference in pancreatic LT\(\beta\) protein expression was observed in terms of distribution and amount of LT\(\beta\) positive cells between type 1 (n=9) and type 2 AIP (n=7) cases. Double-staining with markers for T-cells, B-cells and acinar-cells supported this finding (Supplementary Figure 1C).

Serum analysis of healthy individuals and AIP patients

The observed cytokine, chemokine induction was detectable in serum of AIP patients (Figure 1D; Supplementary Figure 1D). Homeostatic chemokines (e.g. CXCL13) and pro-
inflammatory cytokines (TNFα, IL6) were significantly elevated in sera of AIP patients compared to healthy individuals. We next investigated whether these chemokines are modulated upon corticosteroid treatment in AIP patients (n=10) (Supplementary Table1C). Corticosteroid treatment for 12 weeks failed to change the investigated cytokines and chemokines. Therefore, although corticosteroid-treatment strongly reduced some indicators of inflammation and immune activation (IgG, IgG4) (Supplementary Figure1E), important inflammatory mediators remained elevated, indicating a possible cause for disease re-emergence.

**Generation and characterization of Tg(ELa1-Lta,b)mice**

Data presented above indicate a distinct signature in fibrotic pancreatitis and AIP. Strong LT upregulation and its focal expression on metaplastic acinar cells in AIP patients suggested a link between LT expression and AIP. Hence, we tested whether ectopic LT expression in the exocrine pancreas induces tissue damage and AIP in mice. Thus, double transgenic mice expressing LTα and LTβ (ELa1-Lta,b) in the pancreas under the control of the rat elastase 1 promoter were generated (Supplementary Figure2A) [25].

Tg(ELa1-Lta,b) mice showed elevated levels of amylase and lipase by the age of one month (Figure 2A), indicating tissue damage. Pancreas to body weight ratio was calculated in three, six and twelve month-old mice (Figure 2A). Pancreata were significantly smaller (30-40%) in Tg(ELa1-Lta,b) compared to non-transgenic littermates. Already after 8 weeks focal infiltrating immune cells accumulated in the pancreas, accompanied by ADM (Figure2A). Pancreatitis progressed over time, with strong influx of inflammatory cells and concomitant proliferation of acinar-cells with pronounced tissue damage at 12-22 months of age (Figure 2B; Supplementary Figure4). After 6 months of age organized B- and T-cell zones - indicative of TLT formation - were found. Sporadic acinar-to-goblet cell metaplasia and vasculitis were observed; histo-pathological features commonly found in human CP (metaplasia) or AIP (vasculitis) (Figure 2C).
Chronic pancreatitis can lead to endocrine insufficiency; therefore, we tested whether Tg mice are diabetic. Even though Tg(ELa1-Lta,b) mice remained tolerant during an intraperitoneal glucose tolerance test (IPGTT, Figure 3E), they had to secrete ~ 2-3 fold more insulin than their littermates to support the same glucose clearance.

**Tg(ELa1-Lta,b) mice display a transcriptional signature reminiscent of human AIP**

Next, we investigated chemokine and cytokine expression in pancreata of three-and twelve-month-old transgenic mice. Expression of endogenous Lta and Ltb transcripts increased over time. Similar to human AIP patients, induction of homeostatic chemokines (Cxcl13, Ccl19, Ccl21, Ccl17) was observed. Moreover, elevated expression of Ccl20, the adhesion molecule Vcam, the inflammatory chemokines Cxcl1, Cxcl10, Ccl2, the inflammatory cytokines Baff, Il6, Il1β, Il7 and Tnfα was detected on mRNA and protein level (Figure3A-B). This pattern resembles a progressive inflammatory response concomitant with the formation of a homeostatic environment favourable to TLT development. Similar to human AIP patients, serum protein levels for CXCL13 were elevated in transgenic mice (Figure3C).

To define the immune cell populations in transgenic pancreata at the age of three and six months, we performed flow cytometry on isolated mononuclear cells. Consistent with the histological evaluation, a drastic increase in CD4+, CD8+, B220+CD11b+ cells in transgenic pancreata was found. In contrast, littermates displayed almost no detectable inflammatory cells (Figure3D and Supplementary Figure6B).

In order to elucidate which proinflammatory signaling pathways could be of relevance for AIP development immunohistochemical analysis was performed for nuclear RelA/ RelB - to define activation of the canonical, non-canonical NF-κB signaling, and to analyse activation of Stat3 signaling (Supplementary Figure 6D). We identified RelB translocation mainly in acinar cells of Tg(Ela1-LTab) but not in C57BL/6 mice. In contrast, RelA translocation could not be detected in acinar or infiltrating immune cells. Stat3 phosphorylation was detected in
both acinar and inflammatory cells, indicating a possible involvement of Stat3 signaling in AIP pathogenesis in Tg(Ela1-LTab) mice.

**Germinal center formation and increased regulatory T-cells in Tg(ELa1-Lta,b) mice**

We further characterized the lymphocyte populations in Tg(ELa1-Lta,b) pancreata and in its draining lymph node (mesenteric LN) in more detail. A drastic increase in activation of memory and effector CD4⁺ and CD8⁺ T-cells in Tg(ELa1-Lta,b) pancreata was observed at the age of 12 months. Additionally, we found enhanced recruitment of regulatory T-cells (T_reg) in Tg(ELa1-Lta,b) pancreata (Figure4A-C), which is also described in several autoimmune diseases [26], [27].

Next, we evaluated the T-helper cell subsets (Th1/Th2/Th17) in pancreas, based on their distinct cytokine production profile (Figure4D). Strong upregulation of \( \text{Ifn} \gamma, \text{Tnf} \alpha \) and the transcription factor \( \text{T-bet} \) in 12-month old Tg mice indicates the presence of predominantly Th1-type cells. Although the expression of Gata3 is known to control the development of Th2 lineage, recently a stable Gata3⁺/T-bet⁺ cell subset was described [28]. Lymphotoxin-expressing B cells were recently shown to regulate CXCR5-dependent Th2 response [29]. Correspondingly, cytokines secreted by Th2 cells in Tg mice such as \( \text{Il}4 \) and \( \text{Il}10 \) were also slightly upregulated. Th17 cytokines, \( \text{Il}17A, \text{Il}17F \) and some Th2 cytokines (\( \text{Il}5, \text{Il}13 \)) were undetectable in pancreata of Tg(ELa1-Lta,b) mice (data not shown), which correlates with our analyses of human AIP pancreata. Of note, in peripheral blood of human AIP patients, Th1 cells but not Th17 cells are predominant over Th2-type cells [30].

By the age of three months transgenic pancreata contained HEVs (Figure4E). At later time points follicles developed into TLTs characterized by distinct T- and B-cell areas containing clusters of proliferating lymphocytes, germinal center B-cells, and FDC networks (Figure4F).

*Increased immunoglobulins and auto-antibodies in Tg(ELa1-Lta,b) mice*
We next investigated whether Tg mice display increased immunoglobulins and/or auto-antibodies.

Since IgG4 does not exist in mice, we analysed serum IgG, IgM and IgA levels in twelve-month old Tg(ELa1-Lta,b) mice. We found significant increase in total IgG, IgG1, IgG2b, and IgM of Tg(ELa1-Lta,b) mice (Figure 5A), which parallels the hyper-γ-globulinemia found in human AIP [30].

The increased serum immunoglobulins and the presence of TLTs in the pancreas suggest that Tg(ELa1-Lta,b) mice developed an autoimmune phenotype. Indeed, ANA were present in sera of Tg(ELa1-Lta,b) mice with an incidence of ~66% at the age of twelve months (Figure 5B and Supplementary Figure 8). To assess the specificity of ANA, we measured plasma concentrations of anti-nucleosome and anti-dsDNA antibodies, which were both increased in twelve-month-old Tg(ELa1-Lta,b) mice. Rheumatic factor (RF) is elevated in sera of AIP patients [31], which was also observed in Tg(ELa1-Lta,b) mice along with anti-Smith IgG, indicating systemic autoimmune disease (Figure 5C). Thus, auto-antibodies, as well as indicators of systemic autoimmune disease are present in the sera of Tg(ELa1-Lta,b) mice.

We next investigated whether Tg(ELa1-Lta,b) mice developed auto-antibodies against pancreatic self-antigens, as observed in human AIP [30]. Autoantibodies against PSTI, LF and Lipase were significantly elevated in twelve-month-old Tg(ELa1-Lta,b) mice (Figure 5D).

**Tg(ELa1-Lta,b) T-cells cause acute pancreatic damage in Rag1<sup>−/−</sup> mice**

To examine the immune cells contributing to pancreatic damage in AIP pathogenesis, we transferred C57BL/6 and Tg splenocytes into Rag1<sup>−/−</sup> recipients. 7 days post splenocyte transfer, Tg donor CD3<sup>+</sup> cells, but not B220<sup>+</sup> B-cells (data not shown) were found in pancreata of Rag1<sup>−/−</sup> recipient mice. Concomitantly, significant elevation of serum amylase levels was observed (Figure 5E). In contrast, no changes were observed after adoptive transfer of C57BL/6 splenocytes.
Glomerulonephritis in Tg(ELa1-Lta,b) mice

Transgenic mice developed glomerular lesions at the age of twelve months. The mesangial matrix of Tg(ELa1-Lta,b) kidneys was broadened, resulting in narrowed peripheral capillaries. Mesangial IgG deposits were demonstrated by light- and electron microscopy (Figure 5F; Supplementary Figure 8C). The increased accumulation of immune deposits and Mac-2+ macrophages in glomeruli correlated with age, resemble an immune-complex glomerulonephritis, observed also in some AIP patients. However, in Tg(ELa1-Lta,b) mice we could not observe pathological changes in livers, lungs, salivary – and lacrimal glands (data not shown).

The role of monocytes and lymphocytes in the initiation and progression of AIP

We next crossed Tg(ELa1-Lta,b) mice to mice lacking B- and T-lymphocytes (Rag1-/-) or pro-inflammatory monocytes (Ccr2-/-) [32], [33] (Figure 6A). Crossing to Rag1-/- mice resulted in early pancreatic injury, based on serum lipase levels (Figure 6B) and ADM formation. Furthermore Tg(ELa1-Lta,b)xRag1-/- mice showed increased F4/80+ macrophage infiltration. On the contrary, lack of pro-inflammatory monocytes rescued the early onset of pancreatic tissue damage and prevented lipase elevation; however, modest lymphocytic infiltration was observed. This indicates that pro-inflammatory monocytes support the initiation of pancreatitis in transgenic mice. Altered transcriptional expression at 8 weeks corroborated this difference. Tg(ELa1-Lta,b)xCcr2-/- mice displayed increased homeostatic chemokines, while in the absence of B- and T-cells, genes involved in macrophage activation, e.g. Tnfα, Il6 and Ccl2 were significantly increased (Figure 6C). Between three and six months, B- and T-cells also appear to contribute to pancreatic inflammation, as serum lipase levels show similarly reduced levels in both Tg(ELa1-Lta,b)xRag1-/- and Tg(ELa1-Lta,b)xCcr2-/- mice (Figure 6B). At twelve months, chronic tissue damage appeared to be driven by lymphocytes, as Tg(ELa1-Lta,b)xCcr2-/- show organized T-
and B-cell infiltrates concomitant with significantly elevated serum lipase levels compared to Tg(ELa1-Lta,b)xRag1<sup>-/-</sup> mice. Twelve-month-old Tg(ELa1-Lta,b)xCcr2<sup>-/-</sup> mice displayed elevated anti-PSTI antibody levels (Supplementary Figure10) and immune deposits in kidneys (Figure6D). Thus, lack of pro-inflammatory monocytes protects from early pancreatic damage, but it does not affect the extent of AIP. From three months onwards, lack of lymphocytes resulted in reduced tissue damage and failed to induce AIP, suggesting that lymphocytes are directly involved in sustaining tissue damage and AIP in Tg(ELa1-Lta,b) mice.

**Corticosteroid treatment acts anti-inflammatory but not anti-autoimmune**

Four weeks of prednisolone treatment in twelve-month-old mice significantly reduced histological signs of inflammation and metaplasia (Figure7A-B). However, the systemic autoimmune reaction remained unaffected, with persistence of anti-PSTI antibodies and renal immune complexes (Figure7D-F). Severe side effects of steroid treatment and frequent relapses are well-documented in patients [10]. Therefore, we compared prednisolone treatment with inhibitors of the CD40L-CD40 or the LT<sub>α1β2</sub>-LTβR-signaling pathway (Figure7; Supplementary Figure11). Targeting the CD40 pathway did not result in amelioration of autoimmune pancreatitis in mice. However, LTβR-Ig (a soluble chimeric protein inhibiting LTβR-signaling) [34] abrogated pancreatic inflammation, ADM and significantly reduced expression of chemokines and cytokines compared to prednisolone treatment (Figure7). It led to a reduction of auto-antibodies and immune complex deposition in the glomeruli. This suggests that in Tg(ELa1-Lta,b) mice, prednisolone acts rather anti-inflammatory than anti-autoimmune.
Discussion

The underlying mechanisms inducing and maintaining AIP currently remain elusive. Here, we showed that patients suffering from AIP display high pancreatic LT$\alpha\beta$ and chemokine expression in the inflammatory and fibrotic stages. Not only could we detect expression of LT on infiltrating lymphocytes, but also strong up-regulation on acinar cells that underwent metaplasia. While some aspects of expression was similar in all patients, AIP and severely fibrotic pancreata displayed unique expression signatures.

Existing mouse models for AIP rely on inducing pancreatitis via experimental means i.e. by immunization with self-antigens, injection of pathogens [35] or via adoptive transfer of auto-reactive cells or T$_{reg}$ [36-38]. However, such models can be highly variable. Even though spontaneous AIP development has been reported previously in genetic backgrounds prone to develop autoimmunity [39], a genetic model of AIP with robust penetrance and phenotypic reproducibility on C57BL/6 background did not exist.

Based on our data derived from human AIP patients we have established a novel transgenic mouse model of AIP by expressing LT$\alpha\beta$ specifically on acinar cells. This led to a transcription profile of inflammatory chemokines and cytokines reminiscent of human AIP, causally linking LT expression in the pancreas to AIP development. Ectopic LT expression induced the generation of TLTs - as frequently found in AIP. Surprisingly, localized inflammation in the pancreas developed into AIP and systemic autoimmune disease over time. This coincided with a rise in endogenous pancreatic LT$\alpha\beta$ transcripts, most likely produced by infiltrating immune cells making the inflammatory reaction in the pancreas self-sustaining.

AIP in Tg mice was characterized by (1) development of antibodies against pancreatic self-antigens, (2) presence of ANA, (3) rise in serum IgG, (4) antibodies against commensal bacteria and (5) extrapancreatic manifestation. Formation of pancreatic TLTs, T-cell
polarization (mainly towards Th1), systemic autoimmunity and other organ involvement (e.g. kidney) in Tg mice suggests a disease profile consistent with Type 1 human AIP. The degree of \( \text{LT}_{\alpha\beta} \) transcript up-regulation in transgenic pancreata (50-500x) was comparable to pancreata of human AIP patients (100-200x).

Flow cytometry analysis demonstrated a strong shift in the activation of pancreatic effector and memory T-cells in \( \text{Tg(ELa1-Lta,b)} \) mice. As in other autoimmune diseases, a local increase in \( \text{T}_{\text{reg}} \) cells was observed [40] [26] [41] [42]. TNF has the capacity to expand the functional \( \text{T}_{\text{reg}} \), representing a negative feedback loop to counteract TNF-induced pro-inflammatory effects [43]. This implies that the significantly elevated TNF in 12-month-old transgenic mice correlates with the increase in \( \text{T}_{\text{reg}} \) subset. However, decreased functionality of \( \text{T}_{\text{reg}} \) or \( \text{T}_{\text{eff}} \) resistance on \( \text{T}_{\text{reg}} \)-mediated suppression has been described in human autoimmune diseases [44], which could contribute to AIP development in \( \text{Tg(ELa1-Lta,b)} \) mice.

In \( \text{Tg(ELa1-Lta,b)} \) mice nuclear translocation of RelB was observed, while RelA subunit remained cytoplasmic. This underlines the relevance of non-canonical NF-\( \kappa \)B signaling in the pathogenesis of AIP in \( \text{Tg(ELa1-Lta,b)} \) mice. Furthermore, AIP in \( \text{Tg(ELa1-Lta,b)} \) mice coincided with activation of Stat3 signaling in acinar and inflammatory cells.

The autoimmune phenotype in \( \text{Tg(ELa1-Lta,b)} \) mice could be prevented by genetic depletion of lymphocytes. In contrast, absence of pro-inflammatory (Ly-6C\(^{hi}\)) monocytes identifies them as important contributors to early acinar cell damage. However, lack of Ly6C\(^{hi}\) monocytes did not ablate development of autoimmunity, in contrast to autoimmune models of the CNS [32]. Adoptive transfer of transgenic splenocytes into \( \text{Rag1}^{-/-} \) recipients resulted in influx of T-cells, but not B-cells in pancreata in parallel with acute pancreatic damage. These data suggest that in contrast to the early pancreatic damage mediated by innate immune cells (e.g. pro-inflammatory monocytes), subsequent damage in \( \text{Tg(ELa1-Lta,b)} \) mice is influenced by
adaptive immunecells, particularly T-cells. This is corroborated by the strong reduction of pancreatic tissue damage (e.g. serum lipase) in Tg(ELa1-Lta,b)xRag1⁻ mice between 6-12 months of age. In contrast, Tg(ELa1-Lta,b)xCCR2⁻ mice display similar pancreatic tissue damage when compared to Tg(ELa1-Lta,b) mice.

Thus, acinar-cell specific expression of LT leads to two independent reactions: (1) Early onset of pancreatitis, attracting innate immune cells (e.g. pro-inflammatory monocytes) and activating tissue macrophages, presumably to remove non-viable pancreatic cells. (2) Recruitment of lymphocytes to the pancreas results in further increase in cytokine and chemokine expression contributing to an inflammatory milieu sustaining pancreatitis, metaplasia and enabling germinal center and TLT formation. This environment fosters activation, clonal selection and expansion of auto-reactive lymphocytes in Tg(ELa1-Lta,b) mice.

Currently, the treatment options for AIP are limited. AIP patients respond well to corticosteroid treatment; however disease relapses are frequent. Interestingly, neither pro-inflammatory cytokines nor homeostatic chemokines were reduced upon steroid treatment in sera of human AIP patients, which is likely responsible for the disease relapse. Therefore, a complementary treatment that could additionally down-regulate inflammatory mediators is likely to benefit patients.

Corticosteroid treatment of Tg(ELa1-Lta,b) mice reduced pancreatic inflammation, yet failed to revert the autoimmune repertoire (e.g. anti-PSTI antibodies). In contrast, treatment with LTβR-Ig led to strong decrease of inflammation and also ablated AIP in Tg mice. We also depleted germinal center B-cells using anti-CD40L antibody [45]. Although IgG levels were reduced, the load of pancreatic inflammation and progression to AIP remained unchanged in Tg(ELa1-Lta,b) mice. This could possibly be explained by the presence of long-lived plasma cells in the pancreas that do not respond to anti-CD40L treatment.
While corticosteroid treatment was anti-inflammatory, LTβR-Ig treatment had additional anti-autoimmune effects. Suppression of LTβR-signaling in mice with established AIP protects against peripheral AIP pathologies. Therefore, inhibition of LTβR-signaling pathway could be a viable alternative or supplementary approach for AIP treatment.

In summary, increased local LT expression in the pancreas is sufficient to reproduce various clinical features of human AIP and to induce systemic autoimmunity. Tg(ELa1-Lta,b) mice will facilitate the study of AIP pathogenesis and allow testing potential treatment interventions.
Acknowledgments

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Figure legends:

Figure 1: Characterization of human autoimmune pancreatitis and non-autoimmune pancreatic inflammatory diseases. **(A)** Histological analysis of representative human pancreatic tissues from healthy controls (n=6) (scale bar: 100μm), patients with chronic pancreatitis, severe fibrosis and ductal precipitates (asterisk) (n=12) (scale bar: 200μm) and patients with AIP and vasculitis (arrows; n=16) (scale bar: 100μm). H/E staining shows typical features of pancreatic inflammation (fibrosis) and autoimmune disease (inflammatory infiltrates, vasculitis). **(B)** qPCR analysis of human cryo-material from pancreatic tissues (control n=6, non AIP n=6, AIP n=5) for LTα and LTβ transcripts, homeostatic chemokines and LT target genes (e.g. CXCL13, CCL19, CCL21, CCL17, CCL20) as well as inflammatory mediators (BAFF, TNFα, CCL2, IL6, IL17). **(C)** Immunohistochemical analysis indicates human LTβ expression on inflammatory and acinar cells - especially those undergoing acinar-ductal metaplasia (ADM) (insets). Scale bars: Upper row: Control: 200μm. Severe fibrosis and AIP: 100μm. Lower row: 50μm. **(D)** Serum from healthy controls (n=12) and AIP patients (n=10) - before and after corticosteroid treatment – was analysed for protein expression of CXCL13, CCL19, TNFα and IL6; One-way ANOVA (Kruskal Wallis test with Dunn’s Multiple Comparison Post test).*: P<0.05; **: P<0.01, ***: P<0.001.

Figure 2: Description of pancreatitis in Tg(Ela1-Lta,b) mice. **(A)** Serum lipase and amylase levels are elevated in Tg(ELa1-Lta,b) mice compared to wild type (n=4-7). Changes in pancreas size are visualized by pancreas-to-body-weight ratio with significant decrease in transgenic mice (n=4) compared to wild type (n=5) (P=0.0001, P=0.0028, P=0.0049); Unpaired t-test. **(B)** Development and progression of pancreatic inflammation visualized by H/E staining from 3, 6, 12, 18 and 22 month-old Tg(ELa1-Lta,b) mice compared to age matched negative littermates (scale bar: 100 μm). **(C)** Typical features of chronic pancreatitis were found in Tg(ELa1-Lta,b) mice: ADM (scale bar: 20μm), goblet cell metaplasia (Scale bar: 50μm) and vasculitis (scale bar: 100μm). *: P<0.05; **: P<0.01, ***: P<0.001.
Figure 3: Cellular and molecular characterization of Tg(ELa1-Lta,b) mice. (A) qPCR analysis of pancreatic Lta, Ltb, Cxcl13, Ccl19, Ccl21, Ccl17, Ccl20, Vcam, Cxcl1, Cxcl10, Ccl2, Il6, Il1β, Baff (Tnfsf13b), Il7 and Tnfα transcripts in 3 and 12 month-old Tg(ELa1-Lta,b) pancreata compared to negative littermates (3 and 12 months). (B) Elisa for CXCL13, CXCL10, CXCL1, IL6 and IL1β in pancreas homogenates of C57BL/6 and Tg(ELa1-Lta,b) mice. (C) Serum CXCL13 levels are elevated in 12 month-old Tg(ELa1-Lta,b) (n=10) mice compared to age matched C57BL/6 (n=10) mice. (D) Quantification of CD4⁺, CD8⁺, B220⁺ and CD11b⁺ infiltrating cells at the age of 3 and 6 months in transgenic and wild type mice. (E) Intraperitoneal glucose tolerance test (IP-GTT) of 12 month-old Tg(ELa1-Lta,b) and C57BL/6N mice shows no signs of diabetes in Tg(ELa1-Lta,b) mice, however plasma insulin levels are significantly higher throughout IP-GTT. *: P<0.05; **: P<0.01, ***: P<0.001.

Figure 4: Flow cytometry and immunohistochemical analysis of chronically inflamed Tg(ELa1-Lta,b) pancreata. (A) Flow cytometry analysis for activated CD4⁺, CD8⁺ T-cells (B) regulatory T-cells (FoxP3⁺CD4⁺) in pancreata and mesenteric lymph nodes (mLN) of 12 month-old Tg(ELa1-Lta,b) and C57BL/6 mice. (C) Quantification of activated CD4⁺, CD8⁺ T-cells and regulatory T-cells in pancreata and mLN; unpaired t-test from 3 independent experiments. (D) qPCR analysis of pancreatic Tnfα, Ifnγ, T-bet, Gata3, Il4 and Il10 transcripts in 3 (n=5) and 6 month-old (n=5) Tg(ELa1-Lta,b) pancreata compared to negative littermates (n=5) (3 and 6 months); one-way ANOVA (Kruskal Wallis test with Dunn’s Multiple Comparison Post test). (E) Between 3 and 12 months of age Tg(ELa1-Lta,b) mice develop high endothelial venules (HeV), which are absent from pancreata of age matched C57BL/6 mice (PNAd staining) (scale bar: 100μm). (F) After 6 months of age inflammatory infiltrates are found with distinct CD3⁺ and B220⁺ T- and B-cell zones (scale bar: 200μm). The cluster of Ki67⁺ proliferating inflammatory cells (scale bar: 200μm) is indicative of a germinal center, which is supported by the presence of CD21/35⁺ germinal center B-cells (scale bar: 100μm).
and follicular dendritic cell networks (FDC-M1⁺) (scale bar: 100μm). *: P<0.05; **: P<0.01, ***: P<0.001.

**Figure 5: Detection of antinuclear antibodies (ANA), auto-antibodies against pancreas-specific proteins and immune complex glomerulonephritis.** (A) Serum analysis of 12 month-old Tg(ELa1-Lta,b) shows significantly elevated total IgG, IgG1, IgG2b, IgM and IgA levels compared to negative littermates. (B) Detection of antinuclear-antibodies (ANA) on Hep2 cells with sera obtained from 6, 12 and 18 month-old C57BL/6 and Tg(ELa1-Lta,b) mice (scale bar: 20μm). (C) Transgenic mice possess elevated serum anti-nucleosome, anti-dsDNA antibodies. Rheumatic factor and anti-Smith IgG levels are indicative of a systemic autoimmune disease. (D) Detection of anti-PSTI, anti-lactoferrin and anti-lipase antibodies in sera from 12 month-old Tg(ELa1-Lta,b) and C57BL/6 mice. (E) Representative immunohistochemistry from Rag1⁻/⁻ pancreata after spleen-cell transfer from 12 month-old Tg(ELa1-Lta,b) and C57BL/6 mice (n=3 each). Presence of CD3⁺ T-cells in Rag1⁻/⁻ tissue indicates that autoimmune disease is transferable (scale bars from left to right: 100μm, 200μm, 200μm). Transferred spleen-cells from Tg(ELa1-Lta,b) mice led to pancreatic tissue damage in Rag1⁻/⁻ mice shown by elevated amylase levels. Unpaired t-test was used to compare wild type to transgenic mice. (F) Prominent IgG deposits (arrow) in the mesangium of Tg(ELa1-Lta,b) kidneys compared to controls in which only intravascular IgG is stained. Quantification of IgG deposits in the mesangium and macrophage accumulation in capillaries in 3, 6, 12, 15 and 18 months old Tg(ELa1-Lta,b) (n=5) compared to a pool (n=8) of representative age groups from C57BL/6 mice; unpaired t-test. *: P<0.05; **: P<0.01, ***: P<0.001.

**Figure 6: CCR2⁺ macrophages aggravate the initiation phase of chronic pancreatitis whereas lymphocytes contribute to late damage and autoimmunity.** (A) Immunohistochemical characterization of 2 and 12-month-old Tg(ELa1-Lta,b)xRag1⁻/⁻ and Tg(ELa1-Lta,b)xCcr2⁻/⁻ mice. H/E, F4/80⁺ macrophages, Ki67⁺ proliferating inflammatory and
acinar cells, CD3^\+ T-cells and B220^\+ B-cells are indicated (scale bar: 200μm). (B) Serum lipase levels of 1, 3, 6 and 12 month-old C57BL/6 (n=9); Tg(ELa1-Lta,b) (n=10); Tg(ELa1-Lta,b)xRag1^\+ (n=6), Tg(ELa1-Lta,b)xCcr2^- (n=4) mice; unpaired t-test to compare wild type to Tg(ELa1-Lta,b) mice; One-way ANOVA (Kruskal Wallis test with Dunn’s Multiple Comparison Post test) for comparing Tg(ELa1-Lta,b)xRag1^- and Tg(ELa1-Lta,b)xCcr2^- groups to Tg(ELa1-Lta,b) group . (C) qPCR results from 8 week-old mice (n=4 each) show differences of chemokine and cytokine transcripts (CXCL13, CCL19, CCL21, CXCL1, CXCL9, CXCL10) and genes involved in macrophage activation (TNFα, MCP-1, IL-6, F4/80); One-way ANOVA (Kruskal Wallis test with Dunn’s Multiple Comparison Post test). (D) IgG deposits in kidneys from mice of various genotypes illustrates prominent IgG1 deposits in Tg(ELa1-Lta,b)xCCR2^- mice. (Scale bar 50μm). *: P<0.05; **: P<0.01, ***: P<0.001.

Figure 7: Response to prednisolone or LTβR-Ig treatment in established AIP of Tg(ELa1-Lta,b) mice. (A) Immunohistochemical characterization of pancreatic tissue from 12 month-old mice treated with prednisolone for 4 weeks and with LTβR-Ig fusion protein , compared to age matched untreated Tg(ELa1-Lta,b) mice (scale bar: 200μm). H/E, CD3^\+ T-cells, B220^\+ B-cells, Ki67^\+ proliferating inflammatory cells and acinar cells and F4/80^\+ macrophages are indicated (scale bar: 200μm). (B) Both prednisolone and LTβR-Ig treatment reduces ADM. H/E staining of 12 months old Tg(ELa1-Lta,b) mice untreated (n=5), treated with prednisolone (n=5) and LTβR-Ig (n=5). (scale bar:100μm). ADM was quantified and normalized to mm^2 of pancreatic tissue including control IgG treatment group (n=5). (C) Detection of CXCL10 and CXCL13 protein expression in pancreata of treated (n=5) and control mice (n=3). (D) Detection of anti-PSTI antibodies in untreated Tg(ELa1-Lta,b) mice (n=15) and after treatment with either prednisolone (n=3) or LTβR-Ig (n=4) compared to controls (n=6). (E) Quantification of IgG1 deposits in glomeruli treated with prednisolone (n=3) or LTβR-Ig (n=4) compared to untreated Tg(ELa1-Lta,b) (n=8) and C57BL/6 (n=8)
animals; unpaired t-test. (F) Representative images of IgG deposits in kidneys after LTβR-Ig and prednisolone treatment. Scale bar: 50 μm. *: P<0.05; **: P<0.01, ***: P<0.001.
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