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ORIGINAL ARTICLE

Development of autoimmune pancreatitis is independent of CDKN1A/p21-mediated pancreatic inflammation

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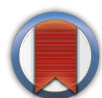

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ABSTRACT

Objective Chronic pancreatitis (CP) and autoimmune pancreatitis (AIP) are characterised by different inflammatory processes. If pancreatic inflammation is a prerequisite for autoimmunity is still unclear. AIP is considered mostly a T cell-mediated disease; however, in induction of CP, macrophages play a pivotal role. p21—a member of cyclin-dependent kinase inhibitors—can influence inflammatory processes, in particular can regulate T cell activation and promote macrophage development. We therefore examined the role of p21-mediated inflammation in AIP.

Design We intercrossed lymphotoxin (LT) overexpressing mice (Tg(Ela1-LTa,b))—a model to study AIP development—with p21-deficient mice. Furthermore, we characterised p21 expression in human AIP and non-AIP specimens.

Results p21 deficiency in LT mice (LTp21$^{-/-}$) prevented early pancreatic injury and reduced inflammation. In acinar cells, diminished proliferation and abrogated activation of non-canonical nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) pathway was observed. In contrast, 12-month-old LT mice with and without p21 had similar inflammatory signatures and T–B cell infiltration. Interestingly, LT and LTp21$^{-/-}$ mice had comparable tertiary lymphoid organs (TLOs), autoantibodies and elevated IgG levels. However, acinar cell proliferation, acinar-to-ductal metaplasia and acinar non-canonical NF-κB pathway activation remained impaired in LTp21$^{-/-}$ pancreata.

Conclusions Our findings indicate that p21 is crucial for pancreatic inflammation in LT-driven pancreatic injury. p21 is involved in early acinar secretion of inflammatory mediators that attract innate immune cells. However, p21 is not essential for humoral immune response, accountable for autoimmunity. Remarkably, p21 renders acinar cells less susceptible to proliferation and transdifferentiation. We therefore suggest that AIP can also develop independent of chronic inflammatory processes.

INTRODUCTION

Autoimmune pancreatitis (AIP) deserves increased awareness as the underlying pathogenesis is not yet fully elucidated.[1] AIP is a progressive disorder, associated with inflammation, metaplasia and fibrosis, but these manifest as distinct clinical histological features.[2–4] Even though inflammatory infiltrates in AIP are mostly localised periductally, the inflammation could specifically attack the acinar cells. IgG antibodies to antigens expressed in pancreatic acinar cells (lactoferrin, pancreatic secretory trypsin inhibitor (PSTI), plasminogen binding protein (PBP), amylase) have been found in the sera of patients with AIP.[5 6] Although AIP is defined as a form of chronic pancreatitis (CP) and recent studies imply that autoimmune pancreatitis can transform into CP,[7 8] there is little agreement on its pathogenesis.

CP is an inflammatory disease of the pancreas characterised by progressive fibrotic destruction of the secretory parenchyma and by duct obstruction leading to functional impairment of both exocrine and endocrine compartments.[1] The inflammation may also attack acinar cells and chronic destruction usually coincides with acinar cell transdifferentiation into duct-like phenotype called acinar-to-ductal metaplasia (ADM).[9]

Cyclin-dependent kinase (CDK) inhibitors are known to regulate inflammatory cell differentiation and function, inflammatory signalling pathways and apoptosis.[10] Therefore, CDK inhibitors are potential candidates to study in inflammatory and autoimmune diseases. CDKN1A/p21, a member of the cip/kip family of endogenous CDK inhibitors, is most importantly involved in arrest of cell cycle progression.[11 12] Further studies revealed that p21 participates in a broader range of biological functions, spanning from the regulation of transcription, apoptosis, DNA repair, cell motility, to stress response, control of cell differentiation and replicative senescence.[13] p21 appears to have a dual-face behaviour in inflammation. The role of p21 as a suppressor of inflammation was highlighted in studies demonstrating that p21 can inhibit macrophage activation[14] and that p21 overexpression can reduce the development of experimental arthritis.[15] Likewise, p21 has been described as a suppressor of autoimmunity in various diseases such as lupus nephritis and rheumatoid arthritis.[16 17] Development of autoantibodies and lupus-like autoimmunity in p21-deficient mice has established that cell cycle deregulation is one of the defective pathways that can lead to loss of tolerance.[18]

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Significance of this study

What is already known about this subject?

- Lymphotoxin is associated with a strong proinflammatory response in human and mouse chronic pancreatitis (CP) and autoimmune pancreatitis (AIP).
- p21—besides its role as a cell cycle inhibitor—is known to confer proinflammatory and proautoimmune properties. It has also been described as a suppressor of autoimmunity in several diseases such as lupus, arthritis and lung inflammation.
- In the pancreas, acinar-to-ductal metaplasia (ADM) development was enhanced in $p21^{-/-}$ mice upon cerulein injection in a mouse model of acute pancreatitis. In this model, the absence of p21 enhanced DNA damage, cellular senescence and altered β-catenin dynamics.
- Currently, the role of cell cycle inhibitors in the pathogenesis of CP and AIP is unknown.

What are the new findings?

- p21 has a proinflammatory role during CP.
- Early accumulation of inflammatory cells and enhanced inflammation in lymphotoxin mice—an established model of AIP—depends on p21.
- p21 interferes with acinar nuclear factor kappa-light-chain-enhancer of activated B cell signalling—thereby modulates the infiltration of immune cells, increases acinar cell proliferation and enhances ADM.
- p21 seems to play a role in the pathogenesis of CP both in human and mouse but has no impact during human or mouse AIP.
- We reveal that CP-associated inflammation is p21 dependent. However, AIP develops in a p21-independent manner.

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

- Cyclin-dependent kinase inhibitor drugs are emerging as potential anti-inflammatory agents that can influence the resolution of inflammation.
- Our results imply that p21 could be a possible therapeutic target in CP but not in AIP.

In contrast, p21 can also potentiate inflammation as $p21^{-/-}$ mice were shown to be resistant to serum transfer-induced inflammatory arthritis,[19] and ablation of p21 in lupus-prone mice led to the reduction of autoimmune disease.[20] Furthermore, loss of p21 ameliorated lung inflammatory responses induced by cigarette smoke or lipopolysaccharide[21] and was protective against atherosclerosis.[22]

We have recently demonstrated a gate-keeper role of p21 in acute pancreatitis, where p21 limits activation of senescence and ADM formation during regeneration.[23] To understand the role of p21 in pancreatic inflammation and autoimmunity, we generated a mouse model by breeding a well-characterised model of AIP (Tg(Ela1-LTa,b))[24] with p21-deficient mice. In the Tg(Ela1-LTab) model, due to the overexpression of lymphotoxin alpha and beta (LTab) on pancreatic acinar cells, a local inflammatory reaction mimics CP at early age (2–3 months) without any presence of autoantibodies. Over time (9–12 months of age), this inflammatory phenotype progresses to AIP with immunological features reminiscent of the human disease, thus making this mouse model a valuable tool to address the role of p21 in pancreatic inflammation and AIP. Therefore, we analysed mice aged 3–12 months old. Our results were corroborated also in human patients.

MATERIALS AND METHODS

Animal husbandry and samples

Animals were maintained under specific pathogen-free conditions. All experiments were approved and conform to the guidelines of the Swiss Animal Protection Law, Veterinary office, Canton Zurich. C57BL/6 and $p21^{-/-}$ B6;129S2-Cdkn1a<tm1Tyj>/J mice were purchased from The Jackson Laboratory. The wild type (wt) controls and all genotype groups analysed in the study were bred on a mixed C57BL/6×129S2 background.

Human samples

Human pancreas biopsies and serum samples were obtained from the University Hospitals Zurich, Oslo and Moscow. All samples were registered in the respective biobanks and kept anonymous. The research project was authorised by the Ethics Committees of the University Hospital Zurich and the Canton of Zurich (Ref. Nr.StV 26–2005). The study protocol was in accordance with the ethical guidelines of the Helsinki declaration.

RNA extraction and real time quantitative reverse transcription PCR (qRT-PCR)

RNA was extracted and used for real-time PCR as described previously . For detailed protocol, please see (Graf 2002) and (Reding 2009) in the online Supplementary file 1.

Serum markers of digestive enzymes produced by the pancreas

Native blood samples for serum analyses were collected by heart puncture and blood cells were precipitated by centrifugation at 7000 rpm for 5 min. Amylase and lipase levels were measured using a serum multiple biochemical analyser (Ektachem DT-System, Johnson & Johnson, Rochester, New York, USA).

Histology and immunohistochemistry

For detailed protocol and list of antibodies, please see the online Supplementary file 1.

Detection of autoantibodies against pancreatic juice proteins

For detailed protocol, please see the online Supplementary file 1.

IgG ELISA

ELISA was performed with a mouse IgG ELISA 'Ready-SET-Go' by eBioscience (8850400) following the manufacturer's instructions. Pancreas homogenates were prepared using Precellys24 dual homogeniser in phosphate-buffered saline-based buffer containing protease inhibitors. The results were normalised to the total protein concentration of the samples.

Statistical analyses and software

GraphpadPrism V.5 (LaJolla, California, USA) was used to construct figures and diagrams. One-way analysis of variance or unpaired t-tests were used where appropriate. In multiple testing, Bonferroni corrections were applied. Differences were considered statistically significant if $p<0.05$ and marked with an asterisk.

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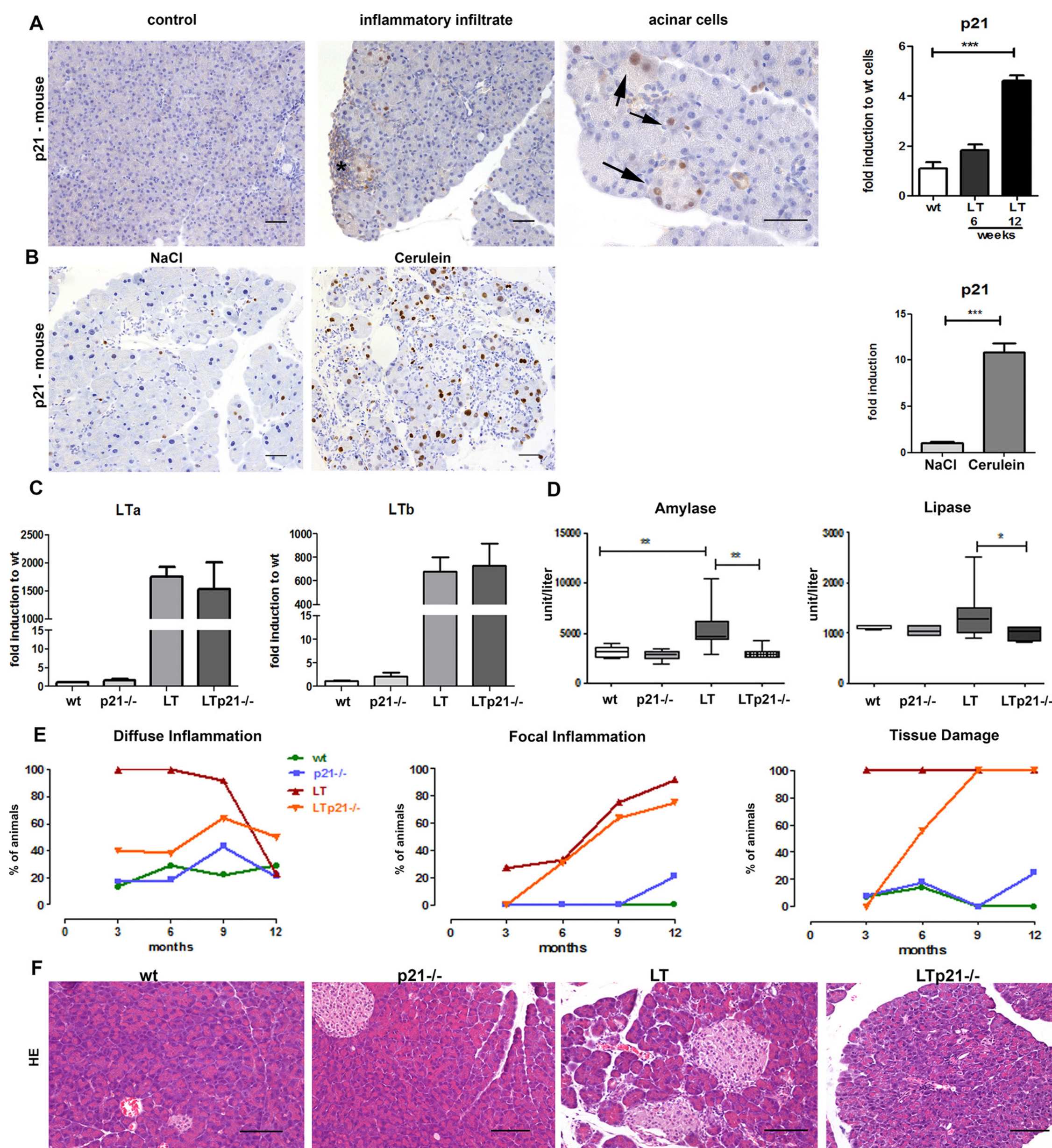

Figure 1 Characterisation of the mouse pancreas of the four genotypes. p21 localisation in a (A) control mouse pancreas and during chronic pancreatic inflammation in 3-month-old lymphotoxin (LT) mice. Asterisk marking area with p21 positive infiltrating cells and arrows pointing towards p21 positive acinar cells (scale bar: 50 µm). p21 mRNA expression in LT mice during pancreatitis. RNA was isolated from cultured acinar enriched cells derived from the whole pancreas. (B) p21 localisation and mRNA expression in cerulein-induced chronic pancreatitis. (C) Gene expression of pancreatic LTa and LTb in 3-month-old mice. (D) Pancreatic injury assessed by serum pancreatic enzyme levels at the age of 3 months. (E) Quantification of pancreatic injury on H&E staining (n=18); animals per group were analysed by a blinded observer. (F) Pancreatic damage visualised on H&E staining in 3month-old animals (scale bar: 50 µm).

RESULTS

LT-induced pancreatic injury is p21 dependent

First, we corroborated p21 expression during pancreatitis in the Tg(ELa1-LTa,b) model and in cerulein-induced CP. Immunohistochemistry confirmed the localisation of p21 to acinar and inflammatory cells (figure 1A and B). p21 expression increased significantly in 3-months-old LT mice and in cerulein-induced CP (figure 1B and C), suggesting a functional involvement of p21 in pancreatitis. To investigate how p21 can modify inflammatory responses during pancreatitis, we crossed Tg(ELa1-LTa,b) LT mice with p21-deficient mice ($p21^{-/-}$) to obtain $LTp21^{-/-}$ mice.

We confirmed that the level of lymphotoxin was comparable in both LT and $LTp21^{-/-}$ groups (figure 1C). The lack of p21 in $LTp21^{-/-}$ mice prevented pancreatic injury as evidenced by normal pancreatic amylase and lipase levels (figure 1D). The progression of tissue damage was evaluated on H&E staining in all four genotypes (figure 1E; see online Supplementary figure 1) by blinded examination. As indicated by the degree of tissue damage at 3 months, the inflammatory phenotype was attenuated in the $LTp21^{-/-}$ group compared with LT mice. At this early time point, LT mice showed predominantly diffuse inflammatory cell infiltration and only few focal infiltrates. In contrast, 3-month-old $LTp21^{-/-}$ animals showed little if any damage to the pancreatic parenchyma (figure 1F). Both

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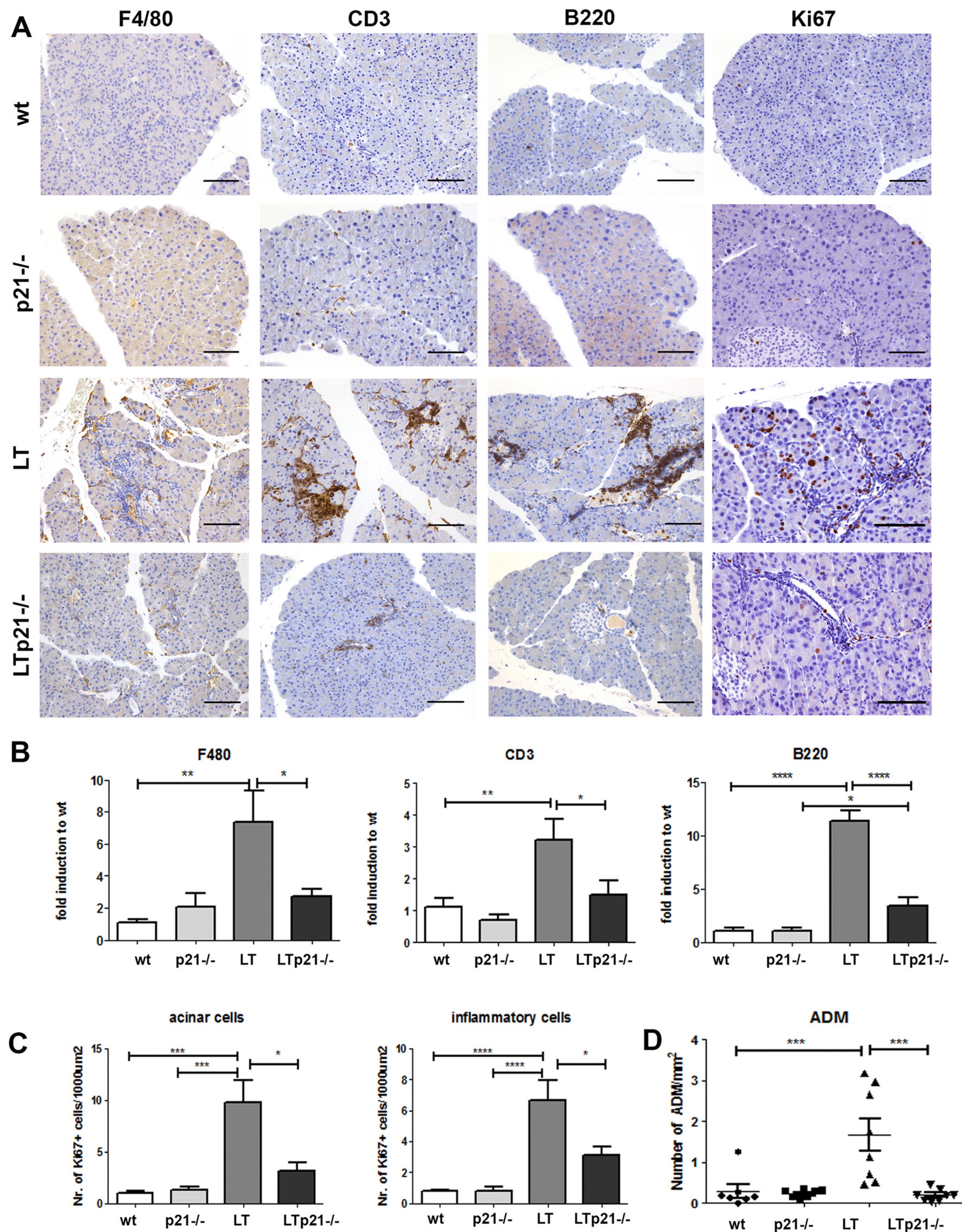

Figure 2 Immunohistochemical analysis of lymphocytes, macrophages and proliferating cells in 3-month-old mice. (A) F4/80+ macrophages, CD3+ T cells, B220+ B cells and Ki67+ proliferating cells are visualised in all four groups, respectively (scale bar: 50 um). (B) Quantification of inflammatory cells in pancreas by quantitative reverse transcription PCR. (C) Quantification of acinar and inflammatory cell proliferation based on Ki67+ staining. (D) Number of acinar-to-ductal metaplasia, quantified on H&E staining and normalised to the total surface of pancreas.

LT and LTp21−/− groups showed increasing focal inflammation at 9 and 12 months. In general, pancreatic injury was significantly reduced in young LTp21$^{-/-}$ mice compared with the LT group, which suggests that p21 is essential for LT-induced pancreatic inflammation.

p21 is critical for LT-mediated inflammatory cell influx and proliferation

Transgenic overexpression of lymphotoxin in the pancreas led to inflammatory cell influx, mostly macrophages and T and B lymphocytes (figure 2A), reproducing previously published findings.[24] In contrast, absence of p21 in LTp21$^{-/-}$ mice significantly decreased the early influx of immune cells (figure 2A and B). The diffuse immune cell infiltration in LT mice was accompanied by extensive proliferation of inflammatory and acinar cells (figure 2C) and consequently by ADM (figure 2D). However, no such proliferative response or ADM was observed in the LTp21$^{-/-}$ pancreas (figure 2C and D). Thus, we assessed the expression of proliferation markers like cyclins and cell cycle inhibitors in 3-month-old mice (see online Supplementary figure 2A). The increased replication in LT mice coincided with upregulation of late phase (A1, B1) cyclins. In contrast, early phase cyclins (D1 and E1) were downregulated in the absence of p21 (p21$^{-/-}$ vs wt and LTp21$^{-/-}$ vs LT), which suggests that lack of p21 downregulates cell proliferation in the pancreas. Therefore, we further evaluated whether the decreased proliferation and downregulation of cyclin D and E correlate with

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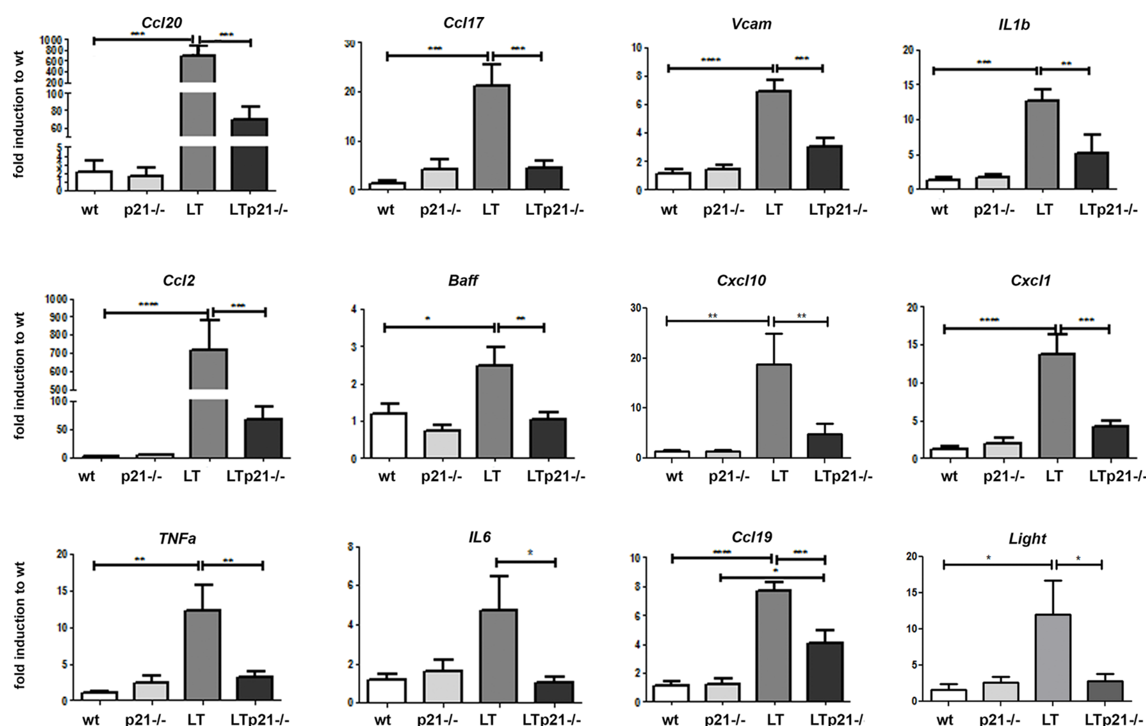

Figure 3 Quantitative reverse transcription PCR analysis at the age of 3 months. *Ccl20, Ccl17, Vcam, Il1b, Ccl2, Baff, Cxcl10, Cxcl1, Tnfa, Il6, Ccl19* and Light transcripts were analysed in mRNA from pancreatic tissue.

a compensatory activation of other cell cycle inhibitors. We did not observe upregulation of p15, p16, p18 or p27 (see online Supplementary figure 2B) suggesting a cell cycle inhibitor independent mechanism to limit acinar cell proliferation.

In summary, inflammatory cell infiltration, acinar cell proliferation and ADM were attenuated in 3-month-old LTp21$^{-/-}$ mice.

p21 determines levels of proinflammatory mediators

Lymphotoxin overexpression represents a strong inflammatory stimulus that induces the release of several proinflammatory mediators.[25] Therefore, we investigated whether LTp21$^{-/-}$ mice exhibit an altered expression profile of representative cytokines and chemokines. Using qRT-PCR, we studied expression levels of LT target genes and pancreatitis-related genes (figure 3). The following proinflammatory genes were expressed significantly less in LTp21$^{-/-}$ pancreata compared with LT mice: *Ccl20, Ccl17, Vcam, Il1b, Ccl2, Baff, Cxcl10, Cxcl1, Tnfa, IL6, Ccl19 and Light* (figure 3) or had a tendency towards lower expression of *Ccl5, Cxcl13* and *Tgfb* (see online Supplementary figure 2C). This implies that the lack of mostly acinar inflammatory mediators in LTp21$^{-/-}$ mice were reducing the inflammatory cell infiltration, especially since receptors potentially activated by LT (LTbR and HVEM) were not significantly regulated (see online Supplementary figure 2C).

p21 modulates non-canonical nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) activation

We have previously demonstrated the relevance of non-canonical NF-κB signalling— mediated by LTbR—in the pathogenesis of AIP in LT mice.[24] To understand the differential inflammatory milieu observed in the pancreas of LT and LTp21$^{-/-}$ mice, we focused on NF-κB pathway, an important modulator of inflammatory cytokine and chemokine expression. We analysed the expression patterns of NF-κB-related proteins in the pancreas. First, nuclear translocation of the canonical NF-κB marker RelA and the non-canonical NF-κB marker RelB was assessed and quantified (figure 4A–C). Acinar cells of LTp21$^{-/-}$ mice showed significantly reduced RelB nuclear translocation compared with LT mice. Notably, infiltrating inflammatory cells in LTp21$^{-/-}$ mice retained their capacity of non-canonical NF-κB activation. Nuclear translocation of RelA was only detected sporadically, indicating no biologically significant activation of canonical NF-κB in these two groups. Moreover, transcripts of A20, a potential negative regulator of canonical NF-κB,[26] were increased in LT mice and unchanged in the LTp21$^{-/-}$ group at 3 months, supporting that canonical NF-κB activation during LT-induced pancreatitis is not essential (see online Supplementary figure 2E). Since LTab, the main ligand of LTbR, is expressed comparably in LT and LTp21$^{-/-}$ mice (figure 1C), we set out to further dissect where the non-canonical NF-κB signalling is impaired between LTbR and p100/p52 processing (figure 4D). NF-κB-inducing kinase (NIK), the most important kinase necessary for RelB translocation,[27] is continuously degraded by the proteasome, mediated by TRAF–cIAP destruction complex.[28–30] We correspondingly observed low NIK levels in wt p21$^{-/-}$ and LTp21$^{-/-}$ mice (figure 4A and C). In contrast, in LT mice, NIK is rescued from degradation. To understand the p21-mediated regulation of NIK, we examined TRAF2/3 or cIAP complexes (see online Supplementary figure 3). Our western blot analysis of TRAF and cIAP shows that TRAF2/3 is unchanged in all experimental groups. However, we noticed significant decrease in cIAP protein only in LT mice. This result suggests that LT expression leads to NIK stabilisation via degradation of cIAP and induces the non-canonical NF-κB signalling. Whereas, in the absence of p21 in LT mice, cIAP was not degraded and kept NIK levels low by an intact TRAF–cIAP destruction complex. Similarly, mRNA transcripts of RelB, NIK p100/p52 and p65 were only increased in LT mice, underlining the impairment of non-canonical NF-κB

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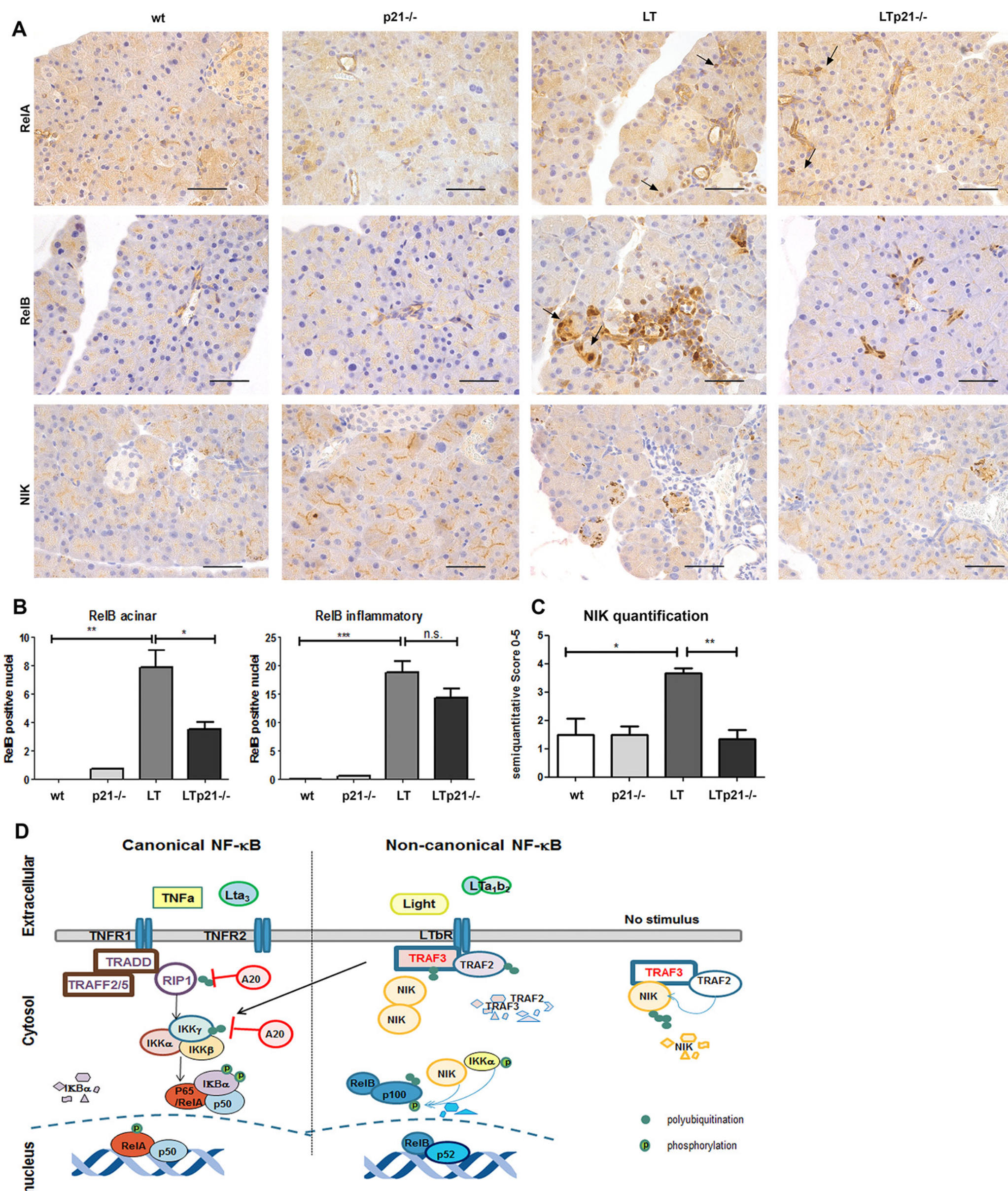

Figure 4 Activation of the nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) pathway in 3-month-old mice. (A) Immunohistochemical analysis of RelA, RelB and NF-κB-inducing kinase (NIK) (scale bar: 50 μm). Arrows indicate nuclear translocation. (B) Quantification of RelB+ nuclei in acinar and infiltrating cells normalised to the surface of pancreas (n=8 animals/group). (C) Quantification of NIK in acinar cells normalised to the surface of pancreas (n=8 animals/group). (D) Canonical activation of NF-κB through the tumour necrosis factor receptors is mediated via the recruitment of tumor necrosis factor receptor-associated cell death domain (TRADD), tumor necrosis factor receptor-associated factor 2 (TRAF2) with receptor interacting protein 1 (RIP1). Polyubiquitination of RIP1 results in recruitment of the inhibitor of kappa B kinase (IKK) complex. IKK phosphorylates IκBα, which triggers its polyubiquitination and subsequent degradation by the proteasome. These events allow translocation of RelA/p50 into the nucleus and activation of gene transcription. Negative regulation of NF-κB is achieved by the ubiquitin-editing enzyme A20, leading to the disassembly of proximal NF-κB-activating complexes and shutting down the inflammatory response. The activation of the non-canonical pathway starts with the stabilisation of the kinase NIK. In the resting state, a destruction complex comprising TRAF2/3 and cellular inhibitor of apoptosis (cIAP), mediates the proteasomal degradation of NIK. Following TRAF recruitment to the receptor, the destruction complex dissociates from NIK, leading to NIK accumulation. NIK then mediates the phosphorylation of p100 and activates IKKa homodimers. Activated IKKa phosphorylates the p100 which results in partial processing by the 26S proteasome and the formation of the p52 subunit. The RelB/p52 dimer then can translocate to the nucleus and induce gene transcription. The activation of the non-canonical NF-κB is limited by the NF-κB-inducible expression of TRAF3 and the inhibitory phosphorylation of NIK by the downstream kinase IKKα.

signalling in the LTp21$^{-/-}$ group (see online Supplementary figure 2E). Thus, p21 plays an important role in the LT-mediated induction of non-canonical NF-κB, specifically in acinar cells. Furthermore, in LTp21$^{-/-}$ mice, non-canonical NF-κB activation is interrupted between LTbR and NIK.

The progressive inflammatory and autoimmune response is independent of p21

As lack of p21 limited the development of pancreatic inflammation at the 3 months' time point, we hypothesised that autoimmunity and the corresponding inflammation will be reduced

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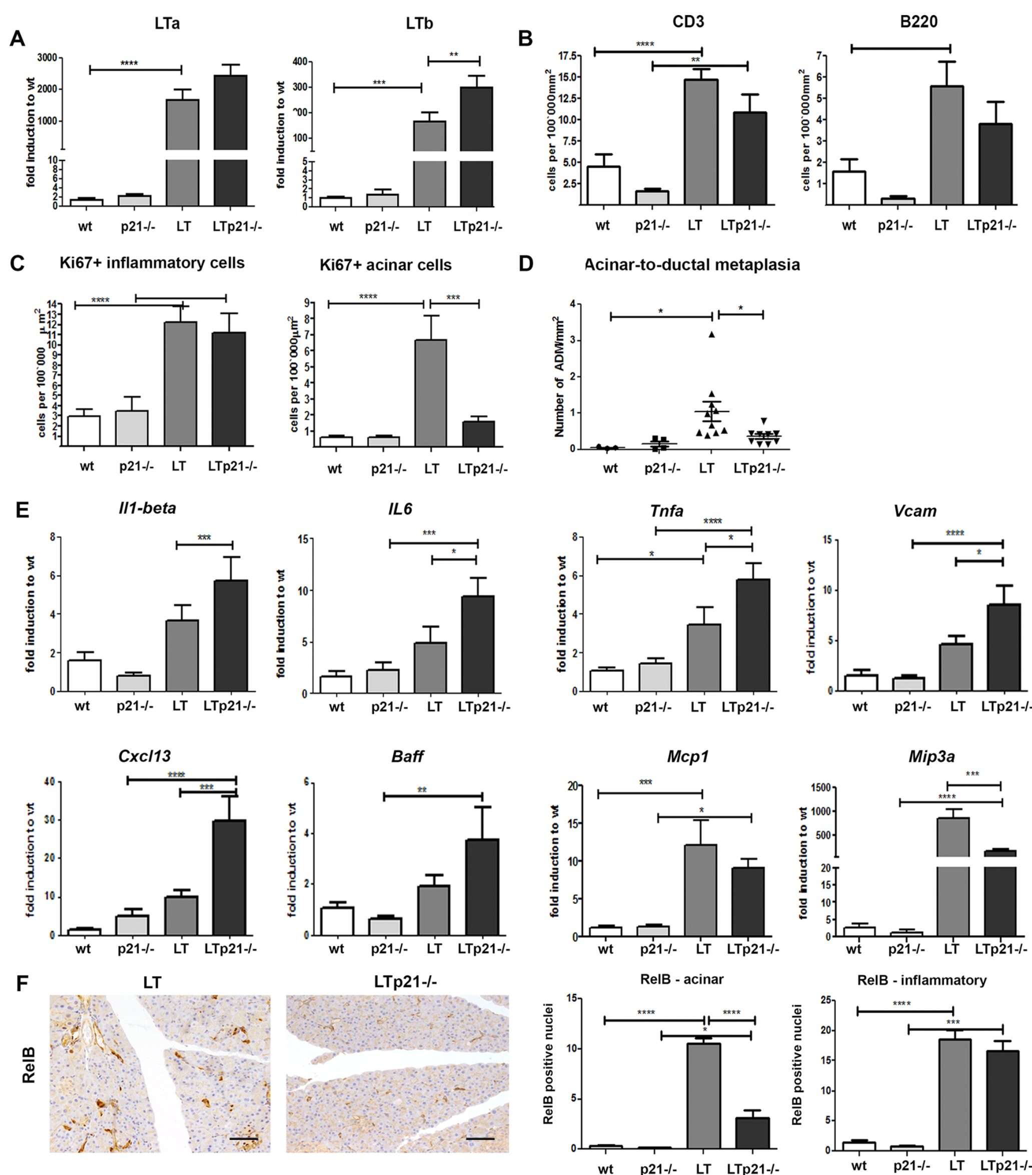

Figure 5 Effects of p21 deficiency on inflammatory cell infiltration, proliferation and NF-κB activation at the age of 12 months. (A) Pancreatic LTab mRNA expression in 12-month-old mice. (B) Number of immune cells: CD3+ T cells, B220+ B cells. (C) Ki67+ proliferating cells are quantified based on immunohistochemistry in all four groups, analysed separately for acinar and inflammatory cells. (D) Number of acinar-to-ductal metaplasia, quantified on H&E staining and normalised to the total surface of pancreas. (E) *Il1b*, *Il6*, *Tnfa*, *Vcam*, *Cxcl13*, *Baff*, *Mcp1* and *Mip3a* gene expression were analysed in mRNA from pancreatic tissue. (F) Immunohistochemical analysis of RelB in LT and LTp21$^{-/-}$ mice (scale bar: 50 µm). Quantification of RelB+ nuclei in acinar and infiltrating cells.

as well. Therefore, we analysed 12 months' time point when LT mice have clear signatures of AIP.[24] The expression levels of the transgenic LTa and LTb transcripts in the LT and LTp21$^{-/-}$ groups were still strongly elevated and in LTp21$^{-/-}$ even higher compared with LT mice (figure 5A). Over time, pancreatic inflammation in LT mice progressed with a strong influx of immune cells and concomitant proliferation of acinar, ductal and inflammatory cells (see online Supplementary figure 4B and D). Unexpectedly, quantitative analysis of infiltrating T and B cells revealed no significant difference between LT and LTp21$^{-/-}$ mice (figure 5B). The same was observed for the proliferative capacity of infiltrating cells, shown by quantification of Ki-67+ cells (figure 5C). The results indicate that in the progressive phase of pancreatitis p21 does not influence the number and proliferation of infiltrating lymphocytes; thus, pancreatic inflammation is comparable in the LT and LTp21$^{-/-}$ groups. qPCR analysis confirmed that LT overexpression and concomitantly increased lymphocyte infiltration is accompanied in both LT and LTp21$^{-/-}$ mice by an upregulation of inflammatory cytokines and chemokines (*Il1b, Il6, Tnfa, Mcp1, Ccl5*), adhesion molecules (*Vcam, Icam*) and homeostatic chemokines (*Ccl19, Ccl20 Mip3a), Cxcl13*) (figure 5E and see figure 5 in the online Supplementary file 1). Particularly, early mediators of inflammation, for example, *Il1b, TNFa*, the adhesion molecule *Vcam* that regulate the migration of B and T lymphocytes, cytokines and chemokines involved in B cell attraction and activation (*Cxcl13, Baff*) were expressed significantly higher in the LTp21$^{-/-}$ mice than in the LT group (figure 5E). The opposite transcriptional signature was observed in genes predominantly produced by macrophages, for example, *Mcp1, Mip3a* (figure 5E). These transcripts showed a trend of higher upregulation in LT mice. The decreased expression of macrophage-derived cytokines is very

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likely linked to the lower number of infiltrating macrophages observed in LTp21$^{-/-}$ mice (see online Supplementary figure 4C). We observed an endogenous reduction of F4/80+ macrophages (p21$^{-/-}$ compared with wt) and decreased macrophage infiltration secondary to inflammatory stimuli (LT vs LTp21$^{-/-}$). To gain a qualitative overview of the macrophage population, we evaluated the subpopulation of M1 and M2 macrophages on mRNA level and immunohistochemistry (see online Supplementary figure 6). In summary, the absence of p21 led to a quantitative reduction of macrophage infiltration, but it did not alter the macrophage phenotype around the follicles and in intact acinar tissue.

p21 contributes to non-canonical NF-κB activation, proliferation and transdifferentiation of acinar cells

While inflammatory cell proliferation was similar, acinar cells proliferated significantly less in the LTp21$^{-/-}$ group compared with LT mice at 12 months of age (figure 5C and see figure 4D in the online Supplementary file 1). Similarly, ADM also remained impaired in LTp21$^{-/-}$ mice (figure 5D). As non-canonical NF-κB signalling can also regulate cell proliferation,[31] we assessed NF-κB activation at this time point as well (figure 5F). The activation of canonical NF-κB remains insignificant (see online Supplementary figure 7A), while non-canonical NF-κB signalling in LT animals was highly activated as published previously.[24] However, RelB nuclear translocation remained significantly lower in acinar cells in LTp21$^{-/-}$ mice, suggesting a regulatory role of p21 on NF-κB activation and proliferation specifically in acinar cells. Interestingly, mRNA expression of RelB was significantly elevated in LTp21$^{-/-}$ pancreata (see online Supplementary figure 7B), indicating that the lack of p21 does not interfere with the transcription of NF-κB related genes.

p21 does not influence LT-mediated AIP development

AIP is described as a T cell-mediated disease; therefore, we investigated if p21 modulates T cell subpopulations. p21-mediated cell cycle regulation of T cells was shown to be essential for establishing tolerance and inhibition of autoimmunity. Data from p21$^{-/-}$ mice suggested a role for p21 in the expansion of activated but not naive T cells.[32] Therefore, we analysed if p21 influences the specific subsets of T helper cells based on the expression of representative transcription factors and cytokines (see online Supplementary figure 5B). Our results reveal no significant difference between LT and LTp21$^{-/-}$ mice. In both groups, Th1 and Th2 responses dominated over Th17 response, consistent with previous reports.[33] This suggests that during AIP, lack of p21 does not influence the effector T cell populations. Additionally, as p21 was shown to influence the accumulation of regulatory T cells,[34] we quantified Foxp3+ cells in the pancreas (see online Supplementary figure 5C). At the 12 months' time point, no significant difference in the number and distribution of regulatory T cells was detected between the LT and LTp21 groups.

Cytokine and chemokine profile obtained from 12-month-old LTp21$^{-/-}$ mice resembled a homeostatic environment favourable to the development of autoimmunity. Therefore, we next characterised tertiary lymphoid follicles (TLOs) that frequently develop during AIP. TLOs are characterised by distinct compartments of T and B cells. They contain clusters of proliferating lymphocytes and CD21/35+ B cells, indicative of germinal centre reaction, as well as FDC-M1+ follicular dendritic cell (FDC) networks. We identified organised inflammatory follicles in both LT and LTp21$^{-/-}$ mice (figure 6A). Quantification of these follicles revealed a lower follicle/area ratio in LTp21$^{-/-}$ mice at 9 and 12 months of age (figure 6B). Further characterisation (figure 6A) revealed that follicles of both LT and LTp21$^{-/-}$ mice harboured the structures reminiscent of TLOs (eg, germinal centres B cells, high endothelial venules and FDC networks). Thus, lack of p21 reduced the extent of follicles but did not prevent the development of presumably functional TLOs. Next, we evaluated if the presence of TLOs is associated with functional autoimmunity, for example, whether the mice exhibited typical hypergammaglobulinemia and autoantibody development. First, we measured IgG production in the pancreas and found that LT and LTp21$^{-/-}$ mice showed similarly elevated values compared with wt mice (figure 6C). Likewise, pancreas-specific autoantibody production remained comparable in LT and LTp21$^{-/-}$ models (figure 6D). These results suggest that the follicles in LTp21$^{-/-}$ mice are fully functional, producing IgGs and autoantibodies similar to LT mice. Furthermore, because deficiency of p21 combined with mild autoreactive backgrounds such as 129/S2×C57BL/6 was shown to lead to development of lupus-like autoimmune glomerulonephritis,[16] we analysed the pathology of kidneys from all four groups (see Supplementary figure 8). We did not notice any lupus-like symptoms in our experimental groups, implying that LTp21$^{-/-}$ did not suffer from additional systemic autoimmune disease.

p21 expression in human pancreatic inflammation

Mouse models do not perfectly mimic the human pathology. Therefore, to validate our findings, we examined p21 expression in human patients. Similar to mouse data, p21 was abundantly expressed on acinar cells including ADM and inflammatory cells during chronic pancreatic inflammation (figure 7A). In contrast to human chronic pancreatic inflammation, analysis of p21 in patients with AIP (figure 7B) revealed only a very low number of p21 positive acinar and inflammatory cells.

The level of p21 mRNA was elevated in patients with chronic pancreatic inflammation and associated with the degree of fibrosis. Corresponding to our findings of p21 staining in patients with AIP, no increase of p21 mRNA was found in this group (figure 7C). These data support our observations in the LT model, suggesting that p21 is not essential in AIP.

Further, we analysed which resident or immune cells contribute to p21 expression during pancreatic inflammation. Immunofluorescence and immunohistochemical analysis revealed co-localisation of p21 with amylase in acinar cells and with CD206 on macrophages. No co-expression is detected in CD3^{+} T cells or aSMA^{+} fibrotic area (figure 7D). Because lymphotoxin is highly expressed during pancreatitis[24] both in acinar and inflammatory cells, we tested whether LT and p21 are co-expressed in human pancreas samples (figure 7E). The staining confirmed previous findings concerning the localisation of LT and p21 on acinar and inflammatory cells; however, co-localisation was not observed.

DISCUSSION

Here we show that p21 selectively enhances pancreatic inflammation, while the lymphocytic autoimmune process is not affected in LT-driven autoimmune pancreatitis.

In addition to its regulatory effect on cell cycle and consequently regeneration,[35] p21 is involved in inflammatory and autoimmune diseases in several models, for example, atherosclerosis, lung inflammation, septic shock, arthritis and lupus.[16 19 21 36] Evidence from these studies suggests that p21 influences proliferation, T cell activation, monocyte differentiation, that is, processes that are involved in the pathophysiology of

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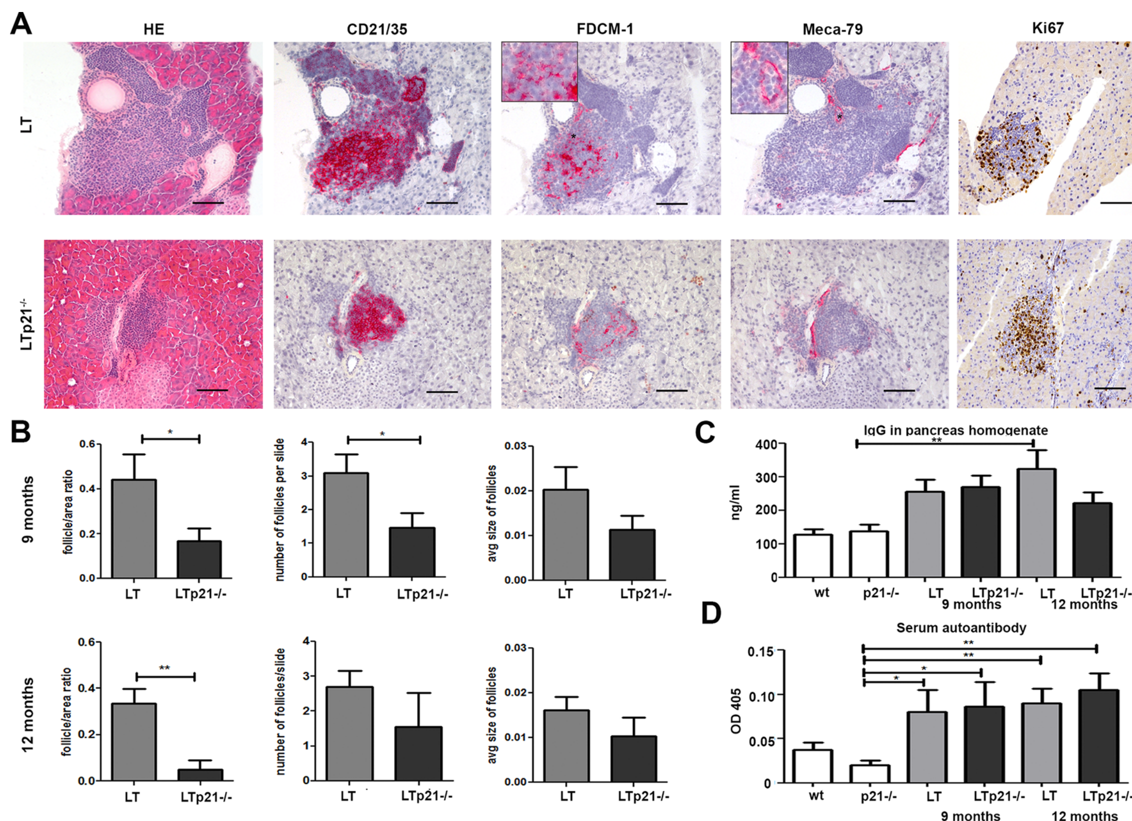

Figure 6 Effects of p21 deficiency on autoimmunity between 9 and 12 months of age. (A) Characterisation of tertiary lymphoid organs by visualising CD21/35+ germinal centre B cells, FDCM-1+ follicular dendritic cell networks, Meca-79+ high endothelial venules and Ki67+ proliferating immune cells in a follicle in 12-month-old mice (scale bar: 50 μm). (B) Total number and size of tertiary lymphoid follicles normalised to the total pancreatic area in mice aged 9 and 12 months. (C) The amount of IgG in pancreas homogenates was determined by ELISA. (D) Autoantibodies against pancreatic juice proteins were measured from the serum of transgenic mice by ELISA.

pancreatitis. Therefore, we examined the role of p21 in pancreatic inflammation and autoimmunity.

The principle finding of this study is a proinflammatory effect of p21 in LT-induced pancreatitis. Our results indicate that absence of p21 affects pancreatic inflammation. Although the inflammatory stimulus, that is, lymphotoxin overexpression is equal in LT and LTp21$^{-/-}$ mice, the transmission of inflammatory response is interrupted in the LTp21$^{-/-}$ group. This was demonstrated by normal serum pancreatic enzyme levels, reduced inflammatory cell infiltration and proliferation, decreased acinar cell proliferation and downregulation of inflammatory gene expression in LTp21$^{-/-}$ compared with LT mice at 3 months of age. It can be hypothesised that absence of p21 affects the survival and homeostasis of inflammatory cells. However, we show that at the age of 12 months, inflammatory cells are able to infiltrate and proliferate in the pancreas and produce inflammatory mediators, without inducing acinar cell proliferation. Furthermore, we have previously shown[23] that during cerulein-induced acute pancreatitis, inflammation was comparable between p21$^{-/-}$ and p21$^{+/+}$ groups, suggesting that inflammatory cells are not affected by the loss of p21. Therefore, our data, and previously published reports,[37] imply the opposite sequence, that is, acinar cells produce cytokines and chemokines, which then drive the inflammatory cell accumulation. Absence of p21 modulates the expression of inflammatory mediators in acinar cells—likely through interference with NF-κB signalling—thereby delaying the infiltration of inflammatory cells.

Interestingly, the effect of p21 deletion on acinar cells was persistent. At the age of 12 months, proliferation of acinar cells and consequently their capacity for ADM were still compromised. This means that acinar cells have either a p21-dependent defective transdifferentiation mechanism, or the phenotype could also be related to the reduced number of macrophages, as the latter are known to promote ADM formation.[9] Our results rather indicate an acinar specific intracellular effect of p21 deletion, based on the impaired activation of non-canonical NF-κB in acinar cells of LTp21$^{-/-}$ mice.

In contrast to our current findings, p21$^{-/-}$ mice were reported to have enhanced ADM development in a model of cerulein-induced pancreatitis.[23] The induction of pancreatitis is fundamentally different in the two models. In the LTp21$^{-/-}$ system, pancreatitis is induced by overexpression of the proinflammatory cytokine lymphotoxin, triggering LTβR which transduces inflammatory signals mainly through the NF-κB pathway. Whereas cerulein—a cholecystokinin (CCK) analogue—primarily acts through Cck1R. Obviously, none of the current mouse models available reflect the human disease fully; yet, both studies revealed important aspects on the role of p21 in the development of pancreatitis. Grabliauskaite *et al*[23] showed that lack of p21 increased DNA damage and cellular senescence in acinar cells, whereas our study suggests an important role of p21 in the LT-mediated induction of non-canonical NF-κB signalling.

NF-κB was previously shown to be influenced by p21.[14 38 39] The induction of p100/p52 processing was dependent on the presence of p21, thereby indicating that p21 functions as

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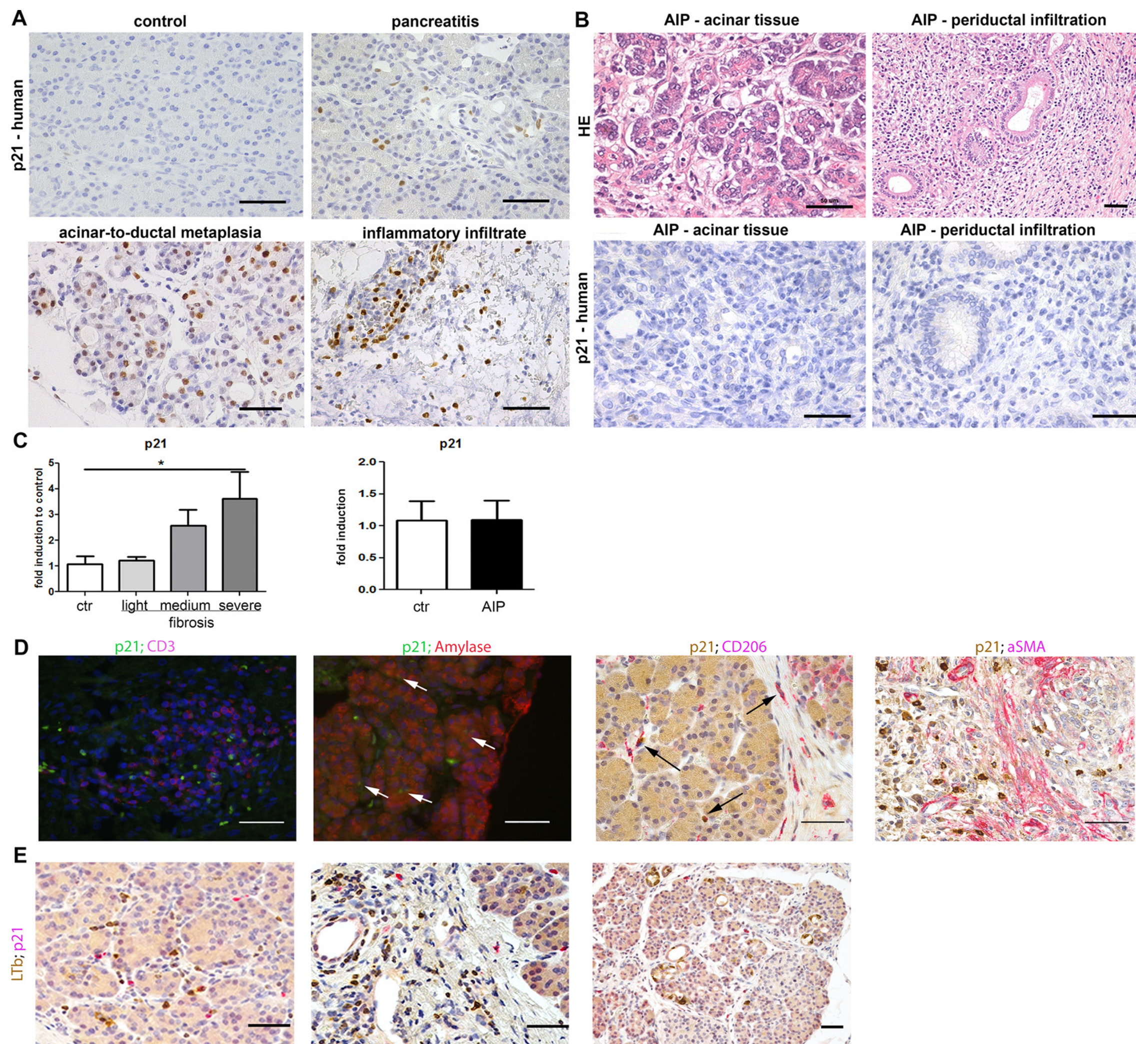

Figure 7 (A) p21 expression in human pancreatic tissue. Samples from healthy control pancreas and different areas representing non-autoimmune pancreatic inflammation, for example, acinar-to-ductal metaplasia and inflammatory cell infiltration (scale bar: 50 µm). (B) p21 distribution during human autoimmune pancreatitis. H&E stains show an area with acinar cells (scale bar: 50 µm) and periductal infiltration (scale bar: H&E 100 µm; p21: 50 µm). (C) p21 mRNA in patients with chronic pancreatic inflammation correlates with the grade of fibrosis (n=minimum six per group). Fibrosis was assessed by Sirius red staining (data not shown). p21 mRNA during human autoimmune pancreatitis (n=5) compared with healthy controls. (D) Double staining of p21 with CD3, amylase, CD206 and aSMA in human chronic pancreatitis. Co-localisation is shown by white and black arrows. (scale bar: 50 µm). (E) Co-staining of LTb and p21 in human tissue from patients with pancreatitis (scale bar: 50 µm).

a modulator in the activation process of non-canonical NF-κB in a rat fibroblast cell line.[40] As such, it is plausible that the reduced activation of NF-κB at 3 months' time point led to decreased acinar expression of inflammatory and homeostatic cytokines and chemokines,[41] which in turn delayed inflammatory cell infiltration. Later, infiltrating cells that were attracted through non-NF-κB-dependent mechanisms contributed to the inflammatory gene expression, inducing further inflammatory cell accumulation. Furthermore, the decreased RelB nuclear translocation in the $LTp21^{-/-}$ acinar cells could be linked to the impairment of proliferation as the non-canonical NF-κB signalling was previously shown to regulate proliferation and survival in various cell types, for example, B cells and plasma cells.[31] Our data therefore suggest that p21 enables LT-induced non-canonical NF-κB activation, as a way of transmitting the inflammatory stimuli, which could have broader implications in various inflammatory syndromes.

Our second important conclusion is that absence of p21 did not change the character of T-cells driving autoimmunity. Deficiency of p21 also did not affect the incidence and timing of LT-driven AIP. $LTp21^{-/-}$ animals developed functional TLOs and they were capable of secreting IgGs and autoantibodies. Furthermore, AIP developed despite a significant decrease of total macrophage numbers and acinar cell proliferation, implying that these processes are dispensable for autoimmunity. Similarly, we already demonstrated in $CCR2^{-/-}$ animals that lack of proinflammatory monocytes did not affect the extent of AIP in LT mice[24] suggesting that macrophages are not critical regulators of AIP development. Our results therefore suggest that the microenvironment created by high LT expression suffice to develop AIP, independent of early pancreatic damage, acinar cell proliferation and macrophage infiltration. We propose that early pancreatic damage is mediated by innate immune cells and acinar cell transdifferentiation, which is modulated by p21. However, humoral immune response, accountable for autoimmunity is not affected.

In summary, the lack of p21 affects acinar cells leading to decreased proliferation concomitant with moderate ADM and reduced activation of non-canonical NF-κB. Reduced acinar cell activation in the absence of p21 decreases overall accumulation of inflammatory cells by delaying recruitment of T and B cells

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and macrophages. However, these strong effects of p21 on early pancreatic inflammation have no influence on AIP, suggesting multiple pathways for AIP development. Consequently, inhibition of p21 might be beneficial for patients with CP or with other diseases in which modulation of the non-canonical NF-kB activation is desired.

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Contributors GS and RG designed and performed the experiments, analysed the data and wrote the manuscript. TR LP and SSt performed the experiments. CV provided human material and participated in paper writing and pathological analysis of tissues and editing the manuscript. SSe contributed with analysis of mouse tissues. AG, SSo, ED and MH participated in editing the manuscript and provided conceptual advice.

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Development of autoimmune pancreatitis is independent of CDKN1A/p21-mediated pancreatic inflammation

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