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Atypical p-ANCA in PSC and AIH: A Hint Toward a "leaky gut"?

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Abstract Primary sclerosing cholangitis (PSC) and autoimmune hepatitis (AIH) are enigmatic chronic inflammatory diseases of the liver, which are frequently associated with chronic inflammatory bowel diseases. Both types of liver disease share various distinct autoantibodies such as atypical perinuclear antineutrophil cytoplasmic antibodies (p-ANCA), and thus are considered autoimmune disorders with atypical features. The discovery that atypical p-ANCA recognize both tubulin beta isoform 5 in human neutrophils and the bacterial cell division protein FtsZ has renewed the discussion on the potential role of microorganisms in the pathogenesis of both diseases. In this paper, we review the evidence for microbial infection in PSC and AIH and discuss new concepts how cross-recognition between microbial antigens in the gut and host components by the immune system along with stimulation of pattern recognition receptors might give rise to chronic hepatic inflammatory disorders with features of autoimmunity.

Keywords Autoimmunity · Antibodies · Autoimmune disease · Infection · Primary sclerosing cholangitis · Autoimmune hepatitis · Toll-like receptor · Regulatory T cells

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Introduction

Primary sclerosing cholangitis (PSC) is an enigmatic cholestatic liver disease of hitherto unknown etiology characterized by progressive inflammatory and fibrosing destruction of intra- and extrahepatic bile ducts eventually leading to liver cirrhosis. PSC shows a frequent association with chronic inflammatory bowel disease (IBD) such as ulcerative colitis and Crohn's disease [1]. In contrast, autoimmune hepatitis (AIH) represents a chronic inflammatory disease of the liver parenchyma characterized by periportal interface hepatitis and predominantly mononuclear necroinflammatory infiltrates. Furthermore, there is also a poorly understood relationship between PSC and autoimmune hepatitis, as both overlap syndromes and clinical transition from frank autoimmune hepatitis to PSC have been observed in individual patients [2, 3]. Finally, PSC must be considered a premalignant condition leading to cholangiocarcinoma in 10–30% of affected patients [4–6] and may also increase the risk of colorectal cancer in patients with IBD [1, 7]. Overall, no therapy has yet proven effective in PSC, and orthotopic liver transplantation remains the only treatment option increasing patient survival.

In the past, close linkage between PSC and IBD made Boden et al. believe that PSC was the result of portal bacteremia secondary to ulcerative colitis [8], and subsequently the same authors reported favorable effects of long-term tetracycline therapy [9]. Later on, their hypothesis was abandoned for several reasons: bacteria were not identified in the portal infiltrates around the bile duct(ule)s, and portal bacteremia was not confirmed in patients with ulcerative colitis. Importantly, efficacy of long-term treatment with tetracylines could not be reproduced [10]. Finally, portal vein phlebitis, a histological hallmark of portal bacteremia, is not a characteristic feature in patients with ulcerative

colitis [11, 12] and inflammatory peribiliary infiltrates mainly comprise mononuclear cells but only few polymorphonuclear cells making, conventional bacterial infection an unlikely scenario. Given the fact that neither the etiology nor the pathogenesis of PSC and AIH have been identified, subsequent attempts to understand both diseases have led to diverse hypotheses. Today, two major competing concepts exist: the first one classifies PSC and AIH as autoimmune diseases, whereas the other one assumes PSC and AIH as an immune-mediated inflammatory disease [13–15]. The hypothesis of autoimmune pathogenesis is supported by the presence of various autoantibodies such as perinuclear antineutrophil cytoplasmic antibodies (p-ANCA) or nuclear antibodies (ANA) and requires loss of tolerance to self-antigens, persistent activation of immune effector mechanisms and a PSC- or AIH-specific autoantigen. However, several clinical features particularly true for PSC, e.g., poor responsiveness to immunosuppressive treatment and male preponderance, are not consistent with the classical concept of autoimmunity, making PSC a putative autoimmune disease with atypical features. Unlike PSC, AIH usually responds well to immunosuppressive therapy rendering an autoimmune process in AIH likely. In contrast, the concept of an immune-mediated chronic inflammatory disease involves interaction between innate and adaptive immune responses resulting in persistent tissue-specific inflammatory infiltrates and release of inflammatory and profibrogenic cytokines. In this paper, we propose that these two major pathogenetic concepts of PSC and AIH need not be mutually exclusive and set forth the idea that identification of cross-reactivity between the microtubular protein β-tubulin isotype 5 and the bacterial cell division protein FtsZ, both acting as antigens of p-ANCA in PSC, may provide a link uniting the competing pathogenetic concepts of persistent inflammation and autoimmunity in PSC.

Diagnostic significance of ANCA in AIH and PSC

Primary sclerosing cholangitis and AIH are both considered autoimmune liver disorders because autoantibodies represent an integral part of the diagnostic armentarium. In both diseases, ANCA are detected at high frequencies.

Antineutrophil cytoplasmic antibodies (ANCA) comprise a family of heterogeneous antibodies, which are directed against different subcellular constituents of human neutrophils or myeloid cells. They have been first detected in patients with systemic vasculitides [16], but later on they have also been found at high prevalence (80–96%) in patients with autoimmune liver disorders, such as AIH or PSC, and/or chronic inflammatory bowel diseases, such as ulcerative colitis [17–25]. To date, indirect immunofluorescence microscopy is widely accepted as the standard method for the detection of ANCA. Ethanol-fixed and/or paraformaldehyde-fixed human neutrophils serve as antigen substrate [26, 27]. Serum endpoint titers of ANCA equal to or greater than 1:20 are considered positive. In general, two distinct staining patterns can be distinguished: "cytoplasmic ANCA (c-ANCA)" characterized by a diffuse granular staining of the cytoplasm that are highly indicative for Wegener's granulomatosis and "perinuclear or *p*-ANCA." The latter class of ANCAs can be further subdivided into so-called "classical" p-ANCA characterized by a fine rim-like staining of the perinuclear cytoplasm that are predominantly found in patients with microscopic polyangiitis and "atypical" p-ANCA showing a broad inhomogeneous rim-like staining of the nuclear periphery associated with multiple intranuclear fluorescent foci (Fig. 1) [27]. Using immune electron microscopy, we were able to demonstrate that these intranuclear fluorescent spots correspond to stained invaginations of the neutrophil nuclear envelope [28, 29]. Accordingly, "atypical" p-ANCA in fact represent antineutrophil *nuclear* antibodies, but not antineutrophil *cytoplasmic* antibodies [30–33].

Unlike classical p-ANCA and c-ANCA that represent valuable diagnostic and therapeutic markers in systemic vasculitides such as Wegener's granulomatosis or microscopic polyangiitis [34], atypical p-ANCA have limited value in the clinical management of patients with AIH and PSC: Serum endpoint titers do not correlate with disease

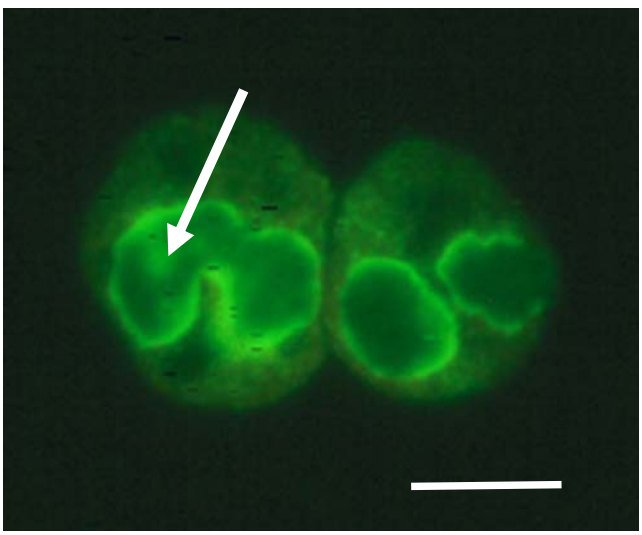

Fig. 1 Microscopic fluorescence pattern of atypical p-ANCA in AIH and PSC. Photographs were taken of ethanol-fixed neutrophilic granulocytes. ANCA were detected with FITC-conjugated goat anti-human IgG-secondary antibodies. The staining pattern of atypical p-ANCA is characterized by a rim-like staining of the nuclear periphery along with multiple intranuclear fluorescent foci. Stained invaginations of the multisegmented nuclei represented the morphologic substrate of the fluorescent intranuclear spots. Serum from a patient with AIH was investigated for the presence of atypical p-ANCA (serum endpoint titer 1:1280). Size bars indicate 10 μm

activity, extent of the disease, or immunosuppressive therapy. In addition, ANCA do not disappear after liver transplantation or colectomy and may even become detectable for the first time after these procedures [35–39]. These puzzling observations have been commonly attributed to the fact that the underlying autoantigen(s) in AIH and PSC were unknown, and it was hoped that identification of the ANCA autoantigens might give rise to improved assays and better understanding of their role in the pathogenesis. Whereas autoantigens of c-ANCA and classical p-ANCA are well-characterized, since almost all c-ANCA-positive sera from patients with Wegener's granulomatosis react with proteinase 3 and myeloperoxidase represents the predominant autoantigen of classical p-ANCA microscopic polyangiitis [16, 40], the autoantigens of atypical p-ANCA remained elusive until very recently. Various proteins have been suggested as potential candidates of atypical p-ANCA in autoimmune liver disorders, including azurocidin, bactericidal/permeability increasing protein, cathepsin G, elastase, lactoferrin [41–49]. However, reactivity to these antigens has only been found in a minority of sera from patients with PSC or AIH (25–35%). As double immunofluorescence staining obtained with sera positive for atypical p-ANCA and antibodies against nuclear antigens suggested a nuclear antigen localization of the antigen rather than the initially proposed reactivity with cytoplasmic proteins [28], nuclear target proteins such as histone H1 [50] and high-mobility non-histone chromosomal proteins 1 + 2 [51, 52] have also been reported as putative target proteins of p-ANCA in PSC and AIH. However, none of the histone proteins shows selective expression in neutrophils, lymphocytes, or biliary epithelial cells.

Finally, we succeeded in identifying a member of the β-tubulin gene family with the closest match to β-tubulin isotype 5 (TBB5) as an ANCA autoantigen reacting with the great majority of sera [53]. Briefly, extracts of nuclear envelope proteins from HL-60 cells were further resolved by two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF) mass spectrometry. The spectrum of identified peptides matched 44% of the acid amino sequence of TBB5 with high probability scores (318 to 780, significant values >45) on the Mascot search engine for rapid protein identification. Detection of a unique fragment at amino acids 283–297 of TBB5 enabled to reliably differentiate this target protein of ANCA from other highly homologous β-tubulin family members listed in the SwissProt data base [54, 55]. To confirm TBB5 as ANCA autoantigen, Cos-7 cells were transiently transfected with human TBB5 cDNA carrying the Xpress sequence tag at the C-terminus. Ninety-four percent of the ANCA-positive sera from patients with AIH and PSC also reacted with recombinant TBB5. Two crucial experiments further confirmed TBB5 as antigen of atypical p-ANCA in AIH and PSC. The specific ANCA-specific immunofluorescence was abolished when ANCA-positive sera had been preabsorbed with tubulin preparations from myeloid cells, whereas ANCA-specific immunofluorescence was enhanced when immunoglobulins in ANCA-positive sera were affinity-purified on myeloid-specific tubulin.

Human TBB5 shares a high degree of structural homology with the bacterial cell division protein FtsZ, an evolutionary precursor of β-tubulin, which is present in almost all bacteria of the intestinal microflora [56]. This fact prompted us to test the hypothesis that ANCA autoreactivity in AIH and PSC might represent cross-recognition of FtsZ with β-tubulin. Thus, FtsZ cDNA of *Escherichia coli M15* with a polyhistidine sequence tag was overexpressed and the gene product was resolved by two-dimensional gel electrophoresis. Reactivity of polyhistidine-tagged FtsZ was detected with 85% of the ANCA-positive sera; ANCA-specific immunofluorescence could be blocked by pre-absorbing sera on recombinant FtsZ.

These novel findings once again invoke a pivotal role of bacteria and the host's antibacterial immune response in the pathogenesis of PSC and AIH. It is important to note that these novel data match with the recently renewed awareness concerning bacteria as a potential cause of AIH and PSC. Such new microbial concepts also comprise the idea that an infectious agent may give rise to antibodies that cross-react with distinct constituents in the host (cross-reactivity or molecular mimicry), interfere with critical pathways of immunoregulation, or induce antibodies that stimulate host cell receptors. Of note, the triggering microorganism no longer needs to be present in these pathogenetic mechanisms once the process has been initiated (hit-and-run concept) [57].

Microbial antigens in AIH and PSC

AIH and PSC are frequently found in association with chronic inflammatory bowel disease. Thus, translocation of bacteria or bacterial antigens into the portal circulation must be considered as a potential cause of bile duct inflammation owing to increased intestinal permeability of the inflamed colon [13, 58, 59]. This idea is particularly supported by animal studies where inoculation of enteric bacteria in the portal vein caused liver inflammation similar to PSC [60]. Furthermore, experimental intestinal bacterial overgrowth in rats resulted in portal inflammation and strictures of the biliary tract [61, 62]. In contrast, a recent human study failed to detect altered intestinal permeability and bacterial overgrowth in patients with PSC [63]. Nevertheless, there is some circumstantial evidence, that microorganisms also cause biliary inflammation and strictures in man. A couple of publications, mainly in patients with immunodeficiency

syndromes, reported AIH- and PSC-like disease in association with the presence of infectious organisms ultimately leading to liver cirrhosis. Incriminated agents comprise various species such as cytomegalovirus, *enterococci*, *brucella*, *cryptosporidia*, *microsporidia*, *candida*, and *trichosporon* species as well as atypical mycobacteria [64–74]. Of note, these hepatobiliary infectious complications are usually not associated with detectable serum autoantibodies and should be referred to as secondary forms of sclerosing cholangitis, although Olsson and coworkers reported a high prevalence of intestinal microorganisms in explanted liver tissue from patients with PSC: positive bacterial cultures were obtained in 21 out of 36 of the explanted livers. However, the results of this study have to be interpreted with caution as detection of bacteria appeared to be correlated to endoscopic interventions performed shortly before liver transplantation [63, 75]. Moreover, secondary bacterial colonization caused by biliary obstruction, altered physicochemical properties of bile as a consequence of chronic inflammation, and bacterial transmigration of the colonic wall in patients with ascites have also to be taken into account.

Thus far, identification of a single causative bacterium inducing PSC remains elusive. However, a large study investigating reactivity of sera from patients with PSC and healthy controls against a panel of 22 viruses, *Chlamydia* species and *Mycoplasma pneumoniae* revealed antibodies of the IgG, IgM, and IgA class against *Chlamydia*-specific lipopolysaccharide as the only immunoserological abnormality associated with PSC. The authors, however, failed to differentiate whether reactivity was directed against *Chlamydia pneumoniae* versus *Chlamydia trachomatis* and concluded that a novel *Chlamydia* species might be involved [57] despite the fact that viable Chlamydia specimens were not detected in liver tissue. Thus, the authors concluded that immunoreactivity to *Chlamydia* lipopolysaccharide in PSC does not reflect ongoing chronic infection but might be a hint that PSC might be initiated by a transient *Chlamydia* infection.

While searching for an infectious agent causing hepatobiliary disorders, *Helicobacter* species seemed a promising candidate. Intestinal *Helicobacter* species were found to enter the circulation especially in immunocompromised patients, and thus could finally be detectable in liver tissue. In animal models, intestinal *Helicobacter* species have convincingly been demonstrated to translocate into the liver, causing chronic hepatic infection associated with lymphocytic necrotizing hepatitis and cholangitis, hepatic adenomas, hepatocellular carcinoma, and cholangiocarcinoma [76–80].

In man, *Helicobacter* species have been frequently detected in bile samples from Korean patients [81]. Moreover, Fox et al. found that patients from Chile with chronic biliary inflammation were commonly infected by bile-tolerant *Helicobacter* species such as *H. hepaticus* and *H. bilis* [82, 83]. Nilsson and coworkers identified gene sequences of *Helicobacter* species by polymerase chain reaction (PCR) in 20 out of 24 liver biopsy samples from patients with PSC and primary biliary cirrhosis, and later on confirmed these data by *Helicobacter*-specific reactivity on immunoblots [84, 85]. This group was also the first to describe that the presence of *Helicobacter* species was associated with particularly high-serum alkaline phosphatase alluding to a potential clinical implication of *Helicobacter* infection in PSC. Finally, morphological intact spiral and coccoid forms of *Helicobacter pylori* have recently been demonstrated by transmission electron microscopy in liver tissue of a single patient with PSC [86]. Taken together, *Helicobacter pylori*, *Helicobacter rodentium*, *Helicobacter pullorum*, *Helicobacter hepaticus*, and *Helicobacter bilis* have been predominantly found among other species. The source of these *Helicobacter* species remains uncertain. It is interesting to note that gene sequences obtained from *Helicobacter*-specific 16s ribosomal DNA (rDNA) is most frequently analogous to *H. pylori* [87, 88]. This observation, along with the fact that most *Helicobacter* species are not present in the portal circulation or in the lymphatics but colonize the gastrointestinal tract, seems to suggest an ascending infection from the duodenum as the most plausible route of infection [89, 90]. The mechanisms that protect *Helicobacter species* against the adverse effects of alkaline pH and bile acids are still a matter of debate [91, 92], but differential expression of virulence factors may enable some *Helicobacter* species, e.g., *H. hepaticus* and *H. bilis*, to become bile-tolerant. In addition, biliary inflammation and biliary obstruction have been shown to markedly decrease bile pH, making colonization by *Helicobacter* secondary to hepatobiliary diseases a possible scenario [93].

Nevertheless, the results of microbial studies in PSC are still conflicting. Most studies relied on PCR-based techniques such as detection of 16S rRNA. As bile acids, intestinal acids, and highly charged mucin components are strong inhibitors of the PCR reaction, results of most studies have to be interpreted with caution. Moreover, immunological assays have not been standardized. In this context, Rudi et al. were unable to detect *Helicobacter* DNA in bile samples from 73 German patients with biliary diseases [94], and seroprevalences of antibodies against *Helicobacter pylori* or *hepaticus* were not significantly raised in sera from patients with autoimmune hepatitis [95]. On the other hand, *Helicobacter*-specific DNA was detected as frequently in controls as in patients with PSC or primary biliary cirrhosis in a study of Boomkens and coworkers [96]. Likewise, Nilsson et al. [97] reported similar frequencies of antibodies against *Helicobacter pullorum*, *H. bilis*, and *H. hepaticus* in patients with PSC and other

autoimmune liver diseases. This lack of disease specificity argues against a role of *Helicobacter* species in the pathogenesis of PSC. Finally, attempts to culture *Helicobacter* from human bile samples have been considerably less reliable than isolation of *Helicobacter* species in experimental animals with Helicobacter-induced liver diseases. Thus, it remains unclear whether detection of *Helicobacter* DNA in bile by molecular techniques reflects enterohepatic circulation of *Helicobacter* species, transient colonization, or gives a hint to actual biliary infection as a cause of PSC.

Despite the aforementioned somewhat controversial results, there is conclusive evidence that antigens from dissociated microbes might trigger autoimmune-like phenomena in PSC as a result of past clinical or subclinical infection. For instance, *Helicobacter* pylori can induce autoantibodies reactive with a protein of the canaliculi in gastric parietal cells, and in a murine model of *H. hepaticus*-induced hepatitis antibodies to heat shock protein (Hsp) 70 were also be detected [98, 99]. Such data provide a basis for molecular mimicry, i.e., microbial molecules share epitopes that cross-react with human autoantigens. The identification of the bacterial cell division protein FtsZ as antigen of p-ANCA in patients with AIH and PSC, and cross-reactivity of p-ANCA with a tubulin isoform of neutrophil granulocytes provides further support for the hypothesis of molecular mimicry between microbial antigens and human autoantigens as a mechanism contributing to the immune-mediated pathogenesis in these diseases [53]. However, FtsZ is highly conserved across a broad range of different microbial species. Thus, identification of FtsZ as a pivotal antigen in PSC does not give a hint to any particular infecting organism. Of note, biliary inflammation may reflect abnormal immune responses to constituents of intestinal microorganisms, which do not necessarily require direct bacterial translocation to the biliary tree or portal circulation. For instance, proinflammatory peptides derived from colonic bacteria were sufficient to induce histological changes resembling PSC in rats with experimental colitis [100, 101]. Such peptides trigger inflammation because they stimulate antimicrobial pattern recognition receptors, e.g., Toll-like receptors (TLR).

Toll-like receptor (TLR) signaling in PSC and AIH

The immune system is endowed with an array of recognition and defense mechanisms capable of responding to foreign factors. These immune responses can be mediated by a set of germline-encoded receptors, such as the Toll-like receptors (TLRs). TLRs extra- and intracellularly recognize the presence of a diverse range of molecular determinants specific

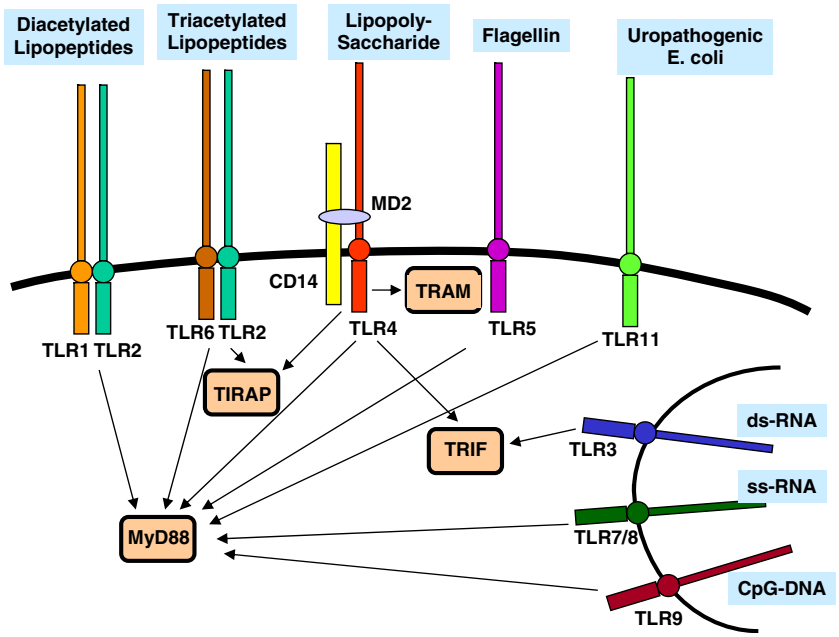

Fig. 2 Toll-like receptors, their ligands, and adaptor molecules. Toll-like receptors (TLR) recognize pathogen-associated molecules. Nucleic acid sensing TLRs 3, 7, 8, and 9 are localized in endosomes. Specificity of TLR signaling is achieved by a couple of distinct adaptor molecules redistributing to the intracellular Toll-IL1 receptor (TIR) domain upon activation: MyD88 (myeloid differentiation factor 88), TIRAP (TIR-domain-containing adaptor protein), TRIF (TIR-domain containing adaptor protein inducing interferon-beta), TRAM (TRIF-related adaptor molecule). Myeloid differentiation protein 2 (MD2) and CD14 are needed to recognize lipopolysaccharide (LPS). Further ligands comprise lipopeptides, flagellin, single-stranded (ss), and double-stranded (ds) RNA and unmethylated cytosine-guanosine DNA motifs (CpG)

to certain microbial pathogens but normally not present in host cells (Fig. 2). Activation of this innate branch of immune defense can lead to various immune responses of different cell types, ranging from production of cytokines, chemokines, costimulatory and adhesion molecules, antimicrobial factors to induction of cell proliferation. Among TLRs, at least 11 different types are known in humans, each one referring to specific microbial ligands (Table 1).

For instance, TLR2 responds to lipoproteins, the main cell wall components of Gram-positive bacteria. TLR2 heterodimerizes with TLR1 and TLR6, enabling discrimination between diacetylated and triacetylated lipopeptides [102]. TLR2/TLR1 heterodimers activate dendritic cells, B lymphocytes, natural killer cells, mast cells, and host endothelial cells [103]. TLR2 and TLR6 collaborate in detecting yeast zymosan [103]. In addition, components of necrotic, but not apoptotic, cells activate fibroblasts and macrophages via TLR2 [104]. Such endogenous ligands have been incriminated as potential culprits both in bacterial and aseptic arthritis [105–107]. However, it cannot be completely ruled out that in these studies autoantigen preparations such as heat shock protein 70 had been inadvertently contaminated by other TLR ligands [108].

TLR4 is a critical component of the lipopolysaccharide (LPS) receptor complex, which activates cells upon exposure to Gram-negative bacteria. However, TLR4 also responds to other ligands. Reports claiming endogenous TLR4 ligands are debated controversially because low-endotoxin preparations of such endogenous molecules failed to confirm the initial observations [108]. Clinically, TLR4 induces sequestration of neutrophil granulocytes in endotoxin-induced lung injury [109], whereas impaired TLR4 signaling can predispose to septicemia in patients with rheumatoid arthritis after anti-TNF therapy [110]. Natural mutants of TLR4 have been identified and are associated with impaired responsiveness to LPS [111], but in man the TLR4 polymorphism does not predispose to rheumatoid arthritis per se [112] and does also not affect the outcome of bacterial sepsis [113].

TLR3, TLR7, TLR8, and TLR9 are located intracellularly in endosomes and are supposed to recognize phagocytosed ligands. TLR3 detects double-stranded (ds)RNA originating from single-stranded (ss) RNA or dsRNA viruses [114, 115]. In addition, TLR3 probably also recognizes secondary RNA structures, because synthetic RNAs, mRNA, and siRNA can similarly trigger production of type I interferons and proinflammatory cytokines. TLR7 and TLR8 both recognize viral ssRNA and distinct synthetic guanosine analogs [103, 116]. TLR3, TLR7, and TLR8, all activate dendritic cells to mature and to produce proinflammatory cytokines [116]. Unmethylated cytosine-guanosine (CpG)-DNA is a stimulatory motif of bacterial and viral DNA, which constitutes an important ligand to trigger TLR9 [117, 118]. The malaria pigment hemozoin, non-CpG DNA, and DNA nanoparticles can also activate TLR9 [119, 120], suggesting that particle-related secondary structures rather than specific sequences are the actual recognition structure. TLR9 resides in the endoplasmic reticulum but redistributes to late endosomes for interaction with ingested CpG-DNA [121]. In man, CpG-DNA is a potent B-cell mitogen; it activates plasmacytoid dendritic cells and, in complex with other proteins, induces

Table 1 Toll-like receptor (TLR) ligands and pathogens

Type of TLR	Microbial Ligand	Endogenous Ligand
TLR1	Cofactor TLR2 and/or TLR4	
TLR2	Lipoteichoic acid (Gram-positive bacteria)	Necrotic cells
	Lipopeptides, LPS (Gram-negative bacteria)	Hyaluronate
	Triacyl lipopeptides (Bacteria; with TLR1)	Fibronectin
	Diacyl lipopeptides (*Mycobacterium* spp., with TLR6)	Heparan sulfate
		Fibrogen, HSPB8
	Lipoarabinomannan (*Mycobacterium* spp.)	
	Glycolipids (*Treponema* spp.)	HSP70
	Zymosan (Fungi)	
	HSP 60 (*Chlamydia trachomatis*)	
TLR3	Double-stranded RNA (Viruses)	Double-stranded RNA
TLR4	Lipopolysaccharides (Gram-negative bacteria)	
	RSV fusion protein (*Saccharomyces cerevisiae*)	
	Mannan (*Candida albicans*)	
TLR5	Flagellin (Gram-positive and Gram-negative bacteria)	
TLR6	Co-factor TLR2	
TLR7 and TLR8 (TLR8 in humans only)	Single-stranded RNA (Viruses)	Single-stranded RNA
TLR9	CpG DNA (all bacteria, viruses)	

RSV respiratory syncytial virus, *HSPB8* heat shock protein B8, *HSP70* heat shock protein 70

strong antigen-specific humoral and cellular inflammatory immune responses of the Th1 type [122].

Taken together, activation of Toll-like receptors by pathogens can activate diverse cell populations of the immune system to initiate or enhance protective B and T cell responses [123, 124]. However, on a susceptible genetic background, TLR signaling induces autoimmunity, and numerous models in experimental animals have meanwhile documented that microbial TLR ligands can trigger a variety of distinct autoimmune diseases such as rheumatoid arthritis, multiple sclerosis (experimental allergic encephalitis in mice), myocarditis, diabetes, and systemic lupus erythematodes [125–130]. Indeed, activation of TLR by microbial agents fulfills several requirements commonly considered necessary to induce autoimmunity:

1) TLR triggering represents a strong stimulus to induce production of interferons and other pro-inflammatory cytokines, thus leading to strong local inflammatory activity.
2) TLR ligands can also act directly or indirectly on CD25-positive regulatory T cells (T_{reg}), which are pivotal to maintain self/non-self discrimination in the immune system [131]. Of note, a study in man described enhanced suppressor function of CD25-positive T_{reg} upon stimulation with the TLR5 agonist flagellin [132]. However, bacterial lipopeptides, which are potent TLR2 agonists, could temporarily suppress the function of T_{reg}, whereas LPS (TLR4 agonist) and CpG (TLR9 agonist) had apparently no effects [133, 134].
3) In the presence of T-cell receptor stimulation, TLR agonists including ligands for TLR2 and TLR9 enhance proliferation and survival of T cells, whereas at the same time they lower the antigenic threshold to trigger antigen-specific T cell activation [135–137].
4) Finally, expression of TLRs 2 and 9 are fine-tuned on polarized intestinal epithelial cells to maintain colonic homeostasis by regulating the balance between tolerance and inflammation, and this delicate balance in TLR expression may become disturbed quite early in the pathogenesis of chronic inflammatory bowel diseases frequently associated with PSC [138].

Thus, engagement of TLRs in the presence of infection and high concentrations of TLR agonists may abrogate suppressor functions of natural CD25-positive T_{reg}, while effector T-cell populations including self-reactive T cells may become expanded. In support of this idea, recent studies suggest that inflammatory infiltrates in PSC contain T cells primed in the gut-associated tissue [139–141]. In two patients with PSC, identical oligoclonality in T-cell receptors was identified when T-cell lines were propagated from biopsies obtained from inflamed common bile ducts 2 years apart [139]. This finding indicates persistent recirculation of T cells to the periductal tissue. Interestingly, the generated T-cell lines proliferated in response to human enterocytes and exhibited enterocyte cytotoxicity. Meanwhile, the hypothesis has been developed from more refined observations that PSC may be caused by long-lived memory T cells primed in the gut, which then migrate to the peribiliary space in response to aberrant expression of gut-specific adhesion molecules and chemokines [140–143].

Our findings of bacterial FtsZ as p-ANCA antigen in PSC nicely supplements the concept of gut-induced immune activation in PSC by providing firm evidence that also B-cell responses are directed against microbial constituents in this disease. A role of intestinal bacteria in the pathogenesis of PSC is further supported by studies in interleukin-10 deficient mice. These mice developed a chronic inflammatory disease of the gut and liver resembling chronic ulcerative colitis along with ANCA-like immunoreactivity if their guts were colonized by a normal intestinal microflora, but remained healthy under germ-free conditions [53, 144, 145]. Sera from mice with normal intestinal microflora reacted with both human β-tubulin and recombinant FtsZ in immunoblots, whereas sera from animals raised under germ-free conditions did not show reactivity with any of the two antigens [53]. This finding corroborates the idea that bacteria are indeed necessary to induce autoimmunity in interleukin-10 knock-

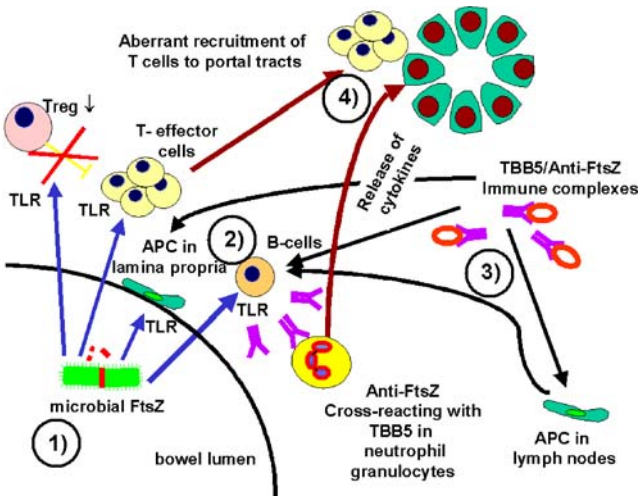

Fig. 3 Role of FtsZ/TBB5 cross-recognition for the pathogenesis of portal inflammation in AIH and PSC. (*1*) In predisposed individuals intestinal microorganisms activate the immune system providing both foreign antigen and stimulation of diverse cells via Toll like receptors. (*2*) The antimicrobial immune response leads to activated T cells and B cells but also antibodies and immune complexes. (*3*) Anti-FtsZ antibodies cross-react with host tubulin beta 5 in neutrophils and give rise to immune complexes consisting of the tubulin beta 5 autoantigen and anti-FtsZ. These immune complexes activate further cells of the immune system and perpetuate the immune response even when the triggering microorganism has meanwhile disappeared. (*4*) T cells primed in the gut carry gut-specific homing signals and are directed toward hepatic portal tracts owing to the aberrant expression of gut-homing receptors and chemokines in this area

out model of chronic inflammatory bowel disease. In these genetically susceptible mice, the microflora provided both a stimulus to trigger TLRs and a microbial structure giving rise to antibodies cross-reactive with a host protein. Both steps are probably needed to induce autoimmunity. For instance, application of CpG-rich oligonucleotides (TLR9 agonist) to transgenic mice expressing the MHC class I molecule Kb exclusively on hepatocytes was sufficient to break tolerance and to induce Kb-specific CD8 positive T cells exerting autoaggression against hepatocytes [146]. Experimental autoimmune hepatitis could be maintained long-term by repeated application of CpG-DNA but subsided after termination of the inflammatory stimulus. Thus, induction of TLR signaling alone appears not be sufficient to maintain autoimmunity. It may be at this step of PSC pathogenesis that antigenic mimicry between microbial and host constituents comes into play. Once triggered in response to a pathogen, continuous activation of self-reactive T and B cells by the self-antigen cross-reacting with a foreign antigen is critically important to maintain autoimmunity [147]. Furthermore, inflammation, insufficient clearance of self-material and immune complex formation between self-antigens and cross-reactive antimicrobial antibodies may result in uptake of circulating self-antigens that are transported to intracellular TLRs expressed in endosomes, thus triggering the vicious circle of immune activation and autoreactivity in PSC and AIH.

In this context, p-ANCA in AIH and PSC might reflect molecular mimicry between bacterial FtsZ and a member of the β-tubulin family (Fig. 3). An abnormal immune response to intestinal microorganisms seems to be the most likely initial step triggering ANCA formation and autoimmunity. Although bacterial infection from intestine is one intriguing possibility, triggering of TLRs by microbial constituents may be sufficient to initiate autoimmunity in a susceptible host. Thus, the gut has to become leaky, not in an anatomical sense but rather in an immunological sense. However, cross-reactivity between immune responses to the causative pathogen and self-antigens may be a pivotal factor to perpetuate the inflammatory process. Finally, reactivity of p-ANCA with a cell division protein abundantly present in intestinal bacteria may explain why chronic inflammatory liver diseases and chronic inflammatory bowel diseases are frequently associated.

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