Dysregulation of signaling pathways associated with innate antibacterial immunity in patients with pancreatic cancer

ROBERT SŁOTWIŃSKI1,2, SYLWIA MAŁGORZATA SŁOTWIŃSKA3

1Department of Surgical Research and Transplantology, Mossakowski Medical Research Centre, Polish Academy of Sciences, Poland
2Department of Immunology, Biochemistry and Nutrition, Medical University of Warsaw, Poland
3Department of Conservative Dentistry, Medical University of Warsaw, Poland

Abstract

Disorders of innate antibacterial response are of fundamental importance in the development of gastrointestinal cancers, including pancreatic cancer. Multi-regulatory properties of the Toll-like receptors (TLRs) (e.g., regulation of proliferation, the activity of NF-κB, gene transcription of apoptosis proteins, regulation of angiogenesis, HIF-1α protein expression) are used in experimental studies to better understand the pathogenesis of pancreatic cancer, for early diagnosis, and for more effective therapeutic intervention. There are known numerous examples of TLR agonists (e.g., TLR2/5 ligands, TLR6, TLR9) of antitumor effect. The direction of these studies is promising, but a small number of them does not allow for an accurate assessment of the impact of TLR expression disorders, proteins of these signaling pathways, or attempts to block or stimulate them, on the results of treatment of pancreatic cancer patients. It is known, however, that the expression disorders of proteins of innate antibacterial response signaling pathways occur not only in tumor tissue but also in peripheral blood leukocytes of pancreatic cancer patients (e.g., increased expression of TLR4, NOD1, TRAF6), which is one of the most important factors facilitating further tumor development. This review mainly focuses on the genetic aspects of signaling pathway disorders associated with innate antibacterial response in the pathogenesis and diagnosis of pancreatic cancer.

Key words: pancreatic cancer, Toll-like receptors, signaling pathways.

Introduction

Pancreatic cancer remains a disease of unknown pathogenesis, and despite advances in diagnosis and the treatment of cancer diseases, it still has a very poor prognosis. It occupies the fourth place as a cause of death from cancer, and the mortality rate from pancreatic cancer is similar to the number of newly detected cases and continues to grow [1-3]. Pancreatic cancer in the early stages of the disease does not give clinical symptoms, making it difficult for early diagnosis that gives hope for a successful therapy. Although there is progress being made in the knowledge on the pathogenesis of pancreatic cancer, only surgery performed in the early stages of the disease can significantly increase the survival. Unfortunately, due to the late diagnosis, only 5-6% of patients with pancreatic cancer survive five years [4-6]. At the time of diagnosis of pancreatic cancer, the tumor is unresectable in 80% of patients and there are already distant metastases; only in a few percent of patients the tumor is in the early stages of development, which in this group (for Stage I invasive cancer with tumors < 2 cm in diameter) may increase the 5-year survival after surgery by 58%, and in patients with tumor < 1 cm even to 100% [7-9]. The median survival reported for the resected pancreatic cancer ranges from 17 to 27 months and the 5-year survival is approximately 20% [10].

A previous study demonstrated that neoadjuvant therapy was associated with improved survival in resectable pancreatic adenocarcinoma, but a recent meta-analysis did not show a significant effect on overall survival among resectable patients after receiving neoadjuvant or adjuvant treatments [11, 12]. The survival time of patients diagnosed with unresectable pancreatic tumor is on average 6 months and may increase only slightly (to 11 months) after the application of a complex chemotherapy (FOLFIRINOX), which confirms the resistance of pancreatic cancer to this type of treatment [13]. Another study reported significantly improved survival in patients with metastatic pancreatic cancer treated with gemcitabine and nab-pacl-
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As demonstrated by previous research, pancreatic ductal adenocarcinoma (PDAC) develops from the most frequent precursors, i.e., pancreatic intraepithelial lesions (pancreatic intraepithelial neoplasia – PanIN), intraductal papillary mucinous neoplasms (IPMNs), mucinous cystic neoplasms (MCNs) and the newly added intraductal tubular papillary neoplasms (ITPNs) [27-31]. PanIN, IPMNs and MCNs lead to invasive PDAC by gene alterations and distant metastasis occurs late during the genetic evolution of pancreatic cancer [32, 33]. For instance, molecular changes in PanINs can be classified as “early” (KRAS mutations, telomere shortening, p21WAF1/CIP1 up-regulation), “intermediate” (cyclin D1 up-regulation, expression of proliferation antigens) or “late” (BRCA2 and TP53 mutations, DPC4/SMAD4/MADH4 inactivation) [32]. Studies in patients with a strong family history of pancreatic cancer revealed a correlation between multifocal neoplastic precursor lesions (PanIN) and lobular atrophy of the pancreas on EUS [34, 35]. Thus, early detection of precursor lesions of the pancreas and surgical removal should significantly improve the results of pancreatic cancer treatment. Our review is aimed at exploring the current knowledge about the pathogenesis and diagnosis of pancreatic cancer based on the genetic aspects of signaling pathway disorders associated with innate antibacterial response.

Innate antibacterial signaling

The discovery of TLRs has enabled a better understanding of disorders of the innate antibacterial response in patients with various diseases, in particular in cancer patients. Toll-like receptors are a family of pattern-recognition receptors, which play a crucial role in the activation of innate and adaptive immunity, and can be expressed in several types of cells, such as macrophages, dendritic cells (DCs), B cells, T cells, monocytes or epithelial cells [36, 37]. TLR proteins recognize a large number of pathogen-associated molecular patterns, such as bacterial lipopolysaccharides or viral RNA. All TLRs, with the exception of TLR3, signal via the MyD88 adapter protein (myeloid differentiation factor 88) [38, 39]. MyD88, TRAF6 (TNF-α receptor-associated factor 6), TRIF (Toll/IL-1-receptor domain-containing adapter inducing interferon) and TRAM (TRIF-related adapter molecule) proteins are key molecules in the cytoplasmic signaling cascade of the antibacterial response initiated by TLRs. TRAF6 is a member of the TNF receptor-associated factor family of proteins and is an E3 ubiquitin ligase, which catalyzes the synthesis of lysine polyubiquitin chain involved in the downstream activation of NF-κB [40]. TLR4-induced TAK1 autophosphorylation and activation require translocation of the MyD88-TRAF6-UBC13-cIAP-TAK1-IKKγ signaling complex from TLR4 into the cytosol, which depends on TRAF6 and cIAPs [41]. The MyD88-dependent pathway involves the early phase of NF-κB activation, which leads to the production of inflammatory cytokines. The MyD88-independent pathway activates interferon (IFN)–regulatory factor (IRF3) and involves the late phase of NF-κB activation, both of which lead to the production of IFN-β and expression of IFN-inducible genes. TLR2 and TLR4 receptors were found to mediate the effects of HMGB1 (high mobility group box-1) in neutrophils and macrophages [42]. HMGB1
is an important protein binding to DNA, stabilizing nucleosomes and facilitating NF-κB activation and gene transcription [43, 44]. HMGB1 modulates the inflammatory cascade in LPS-activated macrophages by inducing the production of pro-inflammatory cytokines TNF-α and IL-1β, while attenuating the release of anti-inflammatory mediators, IL-10 and TGF-β1 [45].

TLRs might influence tumor initiation and progression through regulating the activation of transcription factors, such as NF-κB, interferon regulatory factors (IRFs) or AP-1 via mitogen-activated protein kinase (MAPKs) signaling integrators [46-50]. TLRs caused derangements in several tumor suppressor proteins (such as p16, p21, p27, p53 and pRb), induced STAT3 activation and promoted epithelial-mesenchymal transition (EMT) as well as oncogene-induced senescence [51]. The abnormal expression of TLR receptors may be associated with sepsis and autoimmune diseases (lupus erythematosus, rheumatoid arthritis, type 1 diabetes) [52-56]. Interestingly, TLR receptors have been also detected in many tumor cell lines and tumors, including pancreatic ductal adenocarcinoma, whereas they are not expressed in the normal pancreatic tissue, and may be used as potential therapeutic targets [57-60]. TLRs were found to be involved in tumor cell proliferation, apoptosis and angiogenesis, while the high expression of Toll-like receptor 4/ myeloid differentiation factor 88 was correlated with poor prognosis in patients with colorectal cancer [61-65].

Most studies on pancreatic cancer refer to changes in TLR4 and TLR2 receptor signaling pathways. TLR4 was overexpressed in pancreatic cancer and TLR4 signaling via the MyD88-independent TRIF pathway modulated pancreatic carcinogenesis, because targeting TLR4 or TRIF prevented cancer progression [66]. These findings also suggest that there may be a possible participation of endogenous LPS derived from gut bacteria in modulating pancreatic carcinogenesis. LPS may act through the TLR4-MyD88-NFκB signaling pathway that induces MMP-9 overexpression [67]. As reported by an earlier study, MMP-9 overexpression was related to the progression of pancreatic cancer [68]. Activation of TLR4 signaling by LPS profoundly increased the EMT of pancreatic cancer cells, and M2-polarized TAMS promoted the EMT in pancreatic cancer cells, partially through the TLR4/IL-10 signaling pathway [69]. TLR4 activation was shown to accelerate pancreatic cancer development, while MyD88 blockade using synthetic inhibitors accelerated pancreatic fibro-inflammation and carcinogenesis [70]. More specifically, this meant that MyD88 blockade resulted in an invasive pancreatic adenocarcinoma within 3 weeks in p48Cre;KrasG12D mice treated with caerulein compared with the development of early PanIN lesions in age-matched mice with intact MyD88 signaling. According to these finding, upon MyD88 blockade, DCs seemed to induce the formation of pancreatic antigen-restricted Th2-deviated CD4+ T cells [66]. In that study, stromal leukocytes collected from patients showed increased TLR4 expression, and such up-regulation was also reported both in epithelial and stromal cells in KRAS-mutated mice. Interestingly, inhibition of TLR4 in these mice had a protective effect against tumorigenesis (slower tumor growth) [66]. DCs were found in large quantities in pancreatic cancer and tumor-draining lymph nodes, and the number of tumor-infiltrating TH2 cells inversely correlated with survival in human pancreatic cancer [71]. An in vitro study revealed that the invasive capacity of human pancreatic cancer cells was based on the TLR4-dependent NF-κB activation [72]. Another study correlated the level of TLR4 expression with tumor size, lymph node involvement, venous invasion and pathological stage. Patients with TLR4 overexpression had significantly shorter survival. Lipopolysaccharide (LPS) is a well-established agonist of TLR4 and could be a triggering factor in the initiation and progression of pancreatic cancer [67, 73]. LPS, a putative ligand of TLR4, may promote tumor progression by acting directly on cancer cells and increasing the invasive ability of pancreatic cancer cells through the TLR4/MyD88 signaling pathway [72, 74]. These results also suggest that the TLR4/MyD88/NF-κB signaling pathway plays a significant role in connecting inflammation with cancer invasion and progression. Moreover, silencing of TLR4 or MyD88 completely reversed the effects of LPS [72]. In contrast, the lipid A analogue (OM-174), with a dual stimulating effect on TLR2 and TLR4 receptors, induced regression of several tumor types in animal models [75-77]. Induction of MyD88 and TRIF, downstream of TLR pathways, perturbed cell cycle regulation in pancreatic cancer [70]. Other data demonstrated that protein (mRNA) expression of TLR4, NF-κB p65 and HIF-1α (hypoxia-inducible transcription factor 1α) in pancreatic tumor tissues was increased by 69%, 66% and 70%, respectively, which was significantly higher compared to adjacent normal tissues. This indicated that TLR4 might be partly involved in up-regulating HIF-1α, and they both could synergistically promote the development of pancreatic adenocarcinoma [58]. Another study suggested that TLR4 expression in pancreatic cancer cells was up-regulated via HIF-1α in response to hypoxic stress and underscored the crucial role of HIF-1α-induced TLR4 in tumor growth [78]. Most studies have shown that increased expression of TLR4 is not limited to pancreatic cancer. Toll-like receptors (TLRs) are involved in the development of infectious diseases, autoimmune and neurodegenerative diseases and cancer [79]. TLR4 expression was also detected in head and neck cancer, breast cancer, lung cancer, gastric cancer, hepatocellular carcinoma, colorectal cancer, ovarian cancer, cervical cancer, prostate cancer and melanoma [58, 62]. The results of these studies indicate that the evaluation of TLR4 expression does not differentiate cancerous tumors, which makes diagnosis and treatment difficult.

Toll-like receptor 2 (TLR2) is a promising target for immune system stimulation during cancer immunotherapy and a cell-surface marker for pancreatic cancer [59]. TLR2
stimulation may produce significant antitumor activity by enhancing the innate immunity, T-cell immunity, cytotoxic antibody function and induction of apoptosis [80-82]. Activation of immune response by synthetic lipopeptide (macrophage activating lipopeptide-2 – MALP-2), which activated TLR2 and TLR6 receptors, resulted in a significant tumor growth reduction in a mouse model [83]. Intratumoral injection of the toll-like receptor 2/6 agonist MALP-2, in a limited number of patients with pancreatic carcinoma, caused a decrease in pancreatic tumor proliferation and metastasis [84]. Another agonist of TLR2 receptor protein-bound polysaccharide-K (PSK, mushroom extract) promoted apoptosis and inhibited tumor growth in various human PDAC cell lines [85]. It was found that TLR2 receptors were highly expressed in 70% of pancreatic tumors, but their expression was low in the surrounding normal pancreas tissue; they were also found on tumor-infiltrated myeloid-derived suppressor cells (MDSC) and tumor-associated macrophages (TAM), which after activation promoted tumor expansion, invasion, and metastasis [86]. Interestingly, the disruption of TLR2-dependent antitumor pathways by pancreatic adenocarcinoma up-regulated factor (PAUF protein) could contribute to tumor persistence and facilitate tumor growth [87, 88]. Treatment with anti-recombinant human pancreatic adenocarcinoma up-regulated factor in vitro and in vivo reduced proliferation, migration, invasion, and tumorigenic capacity. These findings suggest that the new cancer-associated ligand, PAUF, may activate the TLR-mediated ERK signaling to produce the pro-tumorigenic cytokines, but it inhibits the TLR-mediated NF-κB signaling, thereby facilitating tumor growth. More specifically, PAUF induces the extracellular signal-regulated kinase (ERK) phosphorylation and activates the IKK-β-mediated TPL2/MEK/ERK signaling pathway through TLR2 and stimulates an increased expression of protumorigenic cytokines (RANTES, MIF). In addition, PAUF was also reported to be associated with the CXC chemokine receptor (CXCR4)-TLR2 complex and to inhibit the CXCR4-dependent, TLR2-mediated NF-κB activation. Another study showed that PAUF could induce the activation and maturation of DCs and activate NF-κB by stimulating the Toll-like receptor signaling pathway [89]. The PAUF-mediated DC activation and immune stimulation are dependent on TLR4 and provide evidence supporting that this protein could be a promising adjuvant for DC-based therapies. The expression of PAUF was also elevated in ovarian tumors and it was found to be more frequently expressed in mucinous adenocarcinoma than in its benign and borderline counterparts, which was associated with a shorter overall survival of patients [90]. Novel high-affinity TLR2 agonists were synthesized to develop targeted agents for cancer imaging and therapy [91]. The latter study demonstrated tumor-specific retention of a fluorescently labeled agonist ligand that could potentially be used for the intraoperative detection of pancreatic cancer cells. Vaccination with TLR2-L SLP conjugates led to the efficient induction of antitumor immunity in mice challenged with aggressive transplantable melanoma or lymphoma [92]. These data show that TLR2-L SLP conjugates are promising synthetic vaccine candidates for active immunotherapy against cancer. Therefore, most of the presented studies, which were aimed at improving the outcomes of pancreatic cancer patient treatment, were focused on the activation of TLR2 receptor signaling pathways.

Fewer studies concerned the TLR3, TLR7 and TLR9 receptors, expressed intracellularly on endothosomal membranes of several types of immune cells (mainly APCs, leukocytes), e.g., macrophages, B cells and DCs [93]. These receptors recognize dinucleotides (CpG motifs) found in bacteria and viruses. The activation of STAT3 and NOTCH receptors through NFκB and MAPK contribute to the aggressive tumor phenotype induced by TLR7 activation. Chimeric mice with Tlr4−/− or Tlr7−/− bone marrows were partially protected from pancreatic carcinogenesis [19, 34]. Furthermore, TLR2, TLR7 and TLR9 were shown to be involved in the initiation of type 1 diabetes mellitus and TLR2, TLR4 played a major role in the onset of type 2 diabetes mellitus. In addition, peroxisome proliferator activated receptor-γ (PPARγ), which is a nuclear receptor transcription factor implicated in the regulation of metabolism, differentiation and inflammation, was also involved in PDAC, which made it an interesting target for pharmacological prevention of PDAC in obese and diabetic patients [94]. TLR7 was found to be highly expressed both in inflammatory (in 40-65% of the tumor microenvironment cells) and cancer cells (in 50% of the advanced tumors) and the role of TLR7 in pancreatic cancer was also reported [51, 90]. For instance, TLR7 was reported to regulate pancreatic carcinogenesis in mice and humans, while TLR7 activation induced a loss of the p16/INK4A gene that is commonly mutated in human PDAC at a relatively early stage [51]. In that study, cancer aggressiveness induced by TLR7 agonists was associated with higher expression of cyclin B1, the c-Myc oncoprotein and the anti-apoptotic Bcl-xL protein. The decrease in cyclin D1, PTEEN, p16 along with the up-regulation of several other tumor-suppressor proteins, such as p53, p21/WAF, pRB or p27/ Kip1 was also observed. Interestingly, both Kras mutations and TLR7 stimulation are necessary for malignant transition. In addition, TLR7 ligation was shown to induce STAT3 activation and interfere with Notch as well as canonical NF-κB and MAP kinase pathways, but the down-regulated expression of Notch target genes and blockade of TLR7 protected against carcinogenesis. Furthermore, mice lacking TLR7 exclusively in their inflammatory cells were protected from neoplasia. The therapeutic mechanism of TLR7 agonist (Imiquimod) was associated with the activation of immune cells involved in an effective antitumor immune response – B lymphocytes, plasma cells, NK and Langerhans cells [95]. Treatment with imiquimod down-regulated
MHC class I molecules on tumor cells and these results indicated that the stimulation of tumor cells with TLR3 or TLR7 ligands enhanced the cytotoxic activity of γδ T cells of cancer patients in vitro [96]. As recently presented in the PDAC cell line, the enhanced Cox-2 expression and increased release of the Cox-2 metabolite, prostaglandin E2 (PGE2), promoted resistance against γδ T cell-mediated lysis; in addition, the application of tribody [(Her2), × Vγ9] that enhances γδ T-cell cytotoxicity, and Cox-2 inhibitors that overcome the PGE2-mediated resistance of PDAC cells to the cytotoxic activity of γδ T cells, might offer a promising combined immunotherapy for pancreatic cancer [97]. A recent study demonstrated that TLR7 and TLR8 were highly expressed by the primary human ductal pancreatic cancer and TLR7/TLR8 expression increased tumor growth in vivo [98]. Stimulation of PANC1 cells overexpressing TLR7/TLR8 resulted in elevated NF-κB and COX-2 expression, increased cancer cell proliferation and reduced chemosensitivity. These data demonstrate stage-dependent up-regulation of both TLR7 and TLR8 expression in pancreatic cancer.

Recently, TLR9 was linked both to acute pancreatitis and cancer [99]. TLR9 was shown to signal through the MyD88-dependent pathway, leading to the production of pro-inflammatory cytokines, but the participation of TLR9 is better known in acute pancreatitis than in pancreatic cancer [100]. The expression of TLR9 was shown to be more pronounced in pancreatic cancer tissue and the TLR9 gene was found to be correlated with the invasive and metastatic potential of human pancreatic carcinoma [101]. The percentage of positive cells expressing TLR9 protein in human pancreatic tissues, paracancerous tissues and normal tissues was 73.3%, 33.3% and 20.0%, respectively. In acute pancreatitis, a similar pattern of staining in exocrine pancreas was reported for TLR4 [102]. On the other hand, TLR2/4/9 agonists seemed to be good candidate molecules prolonging survival in patients with different types of cancer by inducing the expression of tumor necrosis factor α (TNF-α) and inducible nitric oxide synthase (iNOS) that activate dendritic cell trafficking and its associated tumor-specific cytotoxic T-cell responses [103-106]. Evaluation of the efficacy of the ISCOM (immune stimulatory complexes)vaccine in a murine pancreatic carcinoma model revealed that the ISCOM vaccine effectively induced Ag-specific CTL capable of killing tumor cells, but did not affect tumor growth, and this lack of efficacy was correlated with increased numbers of Treg [107]. It should be emphasized that when the authors of the latter study depleted Treg with anti-CD25 mAb, CTL induction and prolonged survival was restored, and adding a low-dose of CpG to the ISCOM vaccine reduced the number of Treg, enhanced CTL responses and initiated regression of pancreatic tumors in a CD8(+)+ T cell-dependent manner. Interestingly, concomitant gemcitabine therapy negatively affected DC vaccine-induced CD8(+)+ T-cell and B-cell responses, but improved clinical efficacy in a murine pancreatic carcinoma model [108]. Despite the reduced number of tumor-reactive T-cells in peripheral blood, in vivo cytotoxicity assays revealed that the cytotoxic T-cell (CTL)-mediated killing was preserved. Another study showed that immunomodulatory oligonucleotide (IMO), TLR9 agonist, interfered with EGFR-dependent signaling (overexpressed in 50-60% of human pancreatic adenocarcinoma), modulating the functional interaction between TLR9 and EGFR, and markedly inhibited the growth of K-Ras mutant colon and pancreatic cancers in vitro [109, 110]. Furthermore, IMO and cetuximab combination caused a potent and long-lasting cooperative antitumor activity in colorectal cancer and in orthotopic pancreatic cancer models in vivo. In an orthotopic mouse model of a human pancreatic tumor xenograft, the antitumor chemotherapy effects (gemcitabine) were combined with the potent TLR9 synthetic agonists (CpG-oligodeoxynucleotides) [111]. These studies showed that the tumor spread in the peritoneal cavity was reduced to a greater extent in combined therapy than with gemcitabine alone, and it significantly improved the survival time.

Considering the fact that the body of evidence is still limited, it is impossible to conclude whether TLR3 and TLR9 play an important role in patients with pancreatic cancer. Similarly as TLR2 and TLR4, TLR3 is expressed in various tumors, including colorectal, ovarian, breast, hepatocellular, laryngeal cancer and melanoma [112-115]. The TLR3 gene codes for an endoplasmic receptor of the pattern-recognition receptors (PRRs) family that recognizes double-stranded RNA (dsRNA) and plays an important role in the innate immune response triggered by viral infection. Several studies demonstrated that activation of TLR3 by dsRNA directly inhibited cell proliferation and induced apoptosis in tumor cells [116-118]. Synthetic dsRNAs (e.g., polyinosine-cytosine, poly I:C; polyadenyl-ic-polyuridylic, poly A:U) were demonstrated to be one of the agonists in tumor chemotherapy demonstrating favorable outcome in clinical trials [119]. TLR3 agonist (polycytidylic acid) was found to induce lysis of pancreatic cancer cell lines by enhancing the cytotoxic activity of γδ T cells in vitro, but this agonist could also acceleratepancreatic carcinogenesis in KRAS-mutated mice [96]. The presence of constitutively activated TLR3/Wnt5a expression in human pancreatic cancer and malignant melanoma cell lines was decreased by phenylethylmethylazole, which in turn inhibited the growth and migration in both of these cancer cell lines [120]. In colon carcinoma, cell line anticancer drugs (e.g., 5-FU) increased the TLR3 expression and intensified the poly I:C-induced apoptosis via p53-dependent and -independent pathways [121]. The results of other studies suggested that IFN-α enhanced TLR3-mediated antiviral cytokine expression in human endothelial and epithelial cells by up-regulating TLR3 expression [122]. Recent data indicated that mucin1 cytoplasmic tail (MUC1-CT), a neg-
ative regulator of TLR inflammatory signaling mediated through the MyD88 adaptor protein, was able to counter-regulate the apoptotic and inflammatory responses of airway epithelial cells through the constitutive association with TLR3, thereby inhibiting poly(I:C)-induced recruitment of TRIF to TLR3 [123]. MUC1 is a membrane-tethered mucin glycoprotein expressed on the apical surface of mucosal epithelial cells and was originally cloned in pancreatic cancer cells [124]. Abruptly glycosylated MUC1 – an oncogenic molecule – is overexpressed in most human epithelial cancers [125]. Overexpression of MUC1 in cancer is caused by the increased gene dosage and the level of transcription, as well as by the loss of post-transcriptional regulation [126]; MUC1 overexpression leads to metastasis and poor prognosis in pancreatic cancer patients [127]. Importantly, by targeting the EGF receptor(s), the STAT1 axis attenuates tumor growth and metastasis through down-regulation of the MUC4 mucin in human pancreatic cancer [128]. In those studies, canertinib or afatinib therapy resulted in a reduction of MUC4 in human pancreatic cancer [128]. In those studies, canertinib or afatinib therapy resulted in a reduction of MUC4 in human pancreatic cancer [128].

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Tumor necrosis factor receptor-associated factor 6 (TRAF6) enabled gastric carcinoma cells to interact with H. pylori. Moreover, other findings suggest that genetic polymorphisms in TLR2 and TLR5 may play important roles in the process of H. pylori-related gastric carcinogenesis [136]. To date, the specific function and detailed mechanism of TLR5 signaling pathways in the development of pancreatic cancer are still unknown.

Among the genes involved in innate antibacterial signaling pathways that affect the development of pancreatic cancer, HMGB1 and TRAF6 are important in the regulation of the local inflammatory response. HMGB1 is one of the most characteristic genes deregulated in solid tumor tissue and serum, including pancreatic cancer [137, 138]. Studies in cell lines showed that HMGB1 was strongly up-regulated in breast cancer, colon cancer, melanoma, pancreatic cancer and prostate cancer; up-regulated HMGB1 activated TLR2 and TLR4 expressed on immune cells and induced cancer progression and metastasis [139]. Moreover, HMGB1 and S100A9 – two DAMPs that bind TLR4 – were detected in the pancreas of PDAC patients [66]. The serum level of HMGB1 showed a tendency to increase with the progression of gastric and pancreatic cancers and is considered a powerful diagnostic biomarker for pancreatic ductal adenocarcinoma [137, 140]. HMGB1 overexpression was associated with tumor progression and poor prognosis in pancreatic cancer and was an independent prognostic indicator for patients’ survival [141]. Overexpression of HMGB1/RAGE (receptor for advanced glycation end products) was associated with reduced apoptosis and prolonged cancer cell survival [142]. The HMGB1/RAGE inflammatory pathway was demonstrated to promote pancreatic tumor growth by regulating mitochondrial bioenergetics [143]. The lack of RAGE, or inhibition of HMGB1 release, diminished the production of ATP and slowed tumor growth in vitro and in vivo. An experimental study showed that TRAF6 (TNF receptor-associated factor 6) acted as an oncogene, which was important for RAS-mediated oncogenesis and provided a mechanistic explanation for the already apparent importance of constitutive NF-κB activation in the RAS-driven lung cancer [144]. The nuclear transcription factor-κB ReIA was also found to be constitutively activated in human pancreatic adenocarcinoma cells [145]. The participation of the TRAF6 gene in the pathogenesis and progression of pancreatic cancer is not clear. Tumor necrosis factor receptor-associated factor 6 is an adapter protein involved in the activation of the TLR-mediated nuclear factor-κB (NF-κB) signaling pathway that induces the production of pro-inflammatory cytokines. TRAF6 as a part of signaling complex (MyD88-IRAK4-IRAK1-TRAF6) is required for the pro-inflammatory cytokine production induced by TLR7 and TLR9 signaling. TRAF6 was shown to up-regulate the expression of hypoxia-inducible factor (HIF-1α) and promote tumor angiogenesis [146]. However, it was demonstrated that poor vascularization was a characteristic feature
of pancreatic cancer [147]. The expression of TRAF6 was up-regulated in pancreatic cancer tissues and over-expression of TRAF6 in pancreatic cancer cells promoted cell proliferation and migration, whereas down-regulation of TRAF6 impaired the tumorigenicity of pancreatic cancer cells in vitro and in vivo [148]. These results suggested that TRAF6 might represent a potential therapeutic target. In an orthotopic xenograft model of SCID mice, a combination of proteasome inhibitor (MG132) and ionizing radiation therapy resulted in a significant increase in the tumor growth delay and a decreased tumor tissue expression of TRAF6 [149]. Additionally, we have recently proposed that over-expression of TLR4, NOD1 and TRAF6 genes and lower MyD88 gene expression in peripheral blood leukocytes of patients with pancreatic cancer, could contribute to chronic inflammation and tumor progression by the up-regulation of the innate antibacterial response (e.g., to LPS) [150]. Although the evidence suggesting carcinogenic and therapeutic effects of stimulation or inhibition of TLRs is increasingly growing, it still remains unclear what kind of changes in gene expression of the TLR signaling pathways in leukocytes are associated with pancreatic cancer.

The results of our study clearly indicated an abnormal gene expression in the innate antibacterial signaling pathways in peripheral blood leukocytes of patients with pancreatic cancer, which might lead to leukocyte dysfunction. We postulate that the pathologic gene expression of receptors and proteins regulating the innate antibacterial response to various stimuli must be primarily included in the analysis of a multistep sequence of events leading to the development and progression of pancreatic cancer. In our opinion, the adjuvant therapy of pancreatic cancer should also focus on the regulation of gene expression in peripheral blood leukocytes. Hypothetically, the influx and recruitment of leukocytes to the tumor microenvironment with the formation of inflammatory infiltration may facilitate cancer cell escape from the control of the immune system in poorly defined mechanisms, for instance, chronic inflammation and immune suppression in the tumor microenvironment with immune responses mediated by Treg, dendritic cells, MDSCs (myeloid-derived suppressor cells) and Th17 cells, MMPs-matrix metalloproteinases and HIF-α-hypoxia-inducible transcription factor overexpression or increased production of IL-10 and H2O2. Changing the microenvironment of the tumor cells by regulating the expression of genes encoding proteins of the innate antibacterial signaling pathway (e.g., using immunonutrition) in leukocytes infiltrating the tumor cells is a novel therapeutic approach (Fig. 1). This applies to both the main tumor mass, which cannot always be removed surgically, and micrometastases that are beyond the reach of diagnostic tests. Furthermore, the assessment of the gene expression profile in leukocytes can be useful for monitoring the course of disease and genetic effectiveness of the treatment. Our previous study in a small group of malnourished pancreatic cancer patients with overexpression of the TLR4 gene in peripheral blood leukocytes revealed that enteral supplementation of glutamine (diet consisting of 20 g glutamine per day) could decrease TLR4 expression within five days prior to surgery [151]. This surprising effect of immunonutrition in pancreatic cancer patients could be associated with a decrease in the amount of stimulants reaching the TLR4 receptor (e.g., by blocking LPS or DAMPs – damage-associated molecular pattern molecules, interacting with TLR4 receptor). An experimental study showed that enteral administration of glutamine reduced the elevated expression of TLR4, signal adaptor protein MyD88 and TRAF6 mRNA in the intestinal mucosa in response to LPS-induced endotoxemia in rats [152]. Investigations on the effect of this treatment on the number of infectious complications and course of the disease are in progress. It is also known that fatty acids (omega-3) can have a selective inhibitory effect on the signaling cascade related to the innate antibacterial response by blocking the interaction of endotoxin with the TLR4 receptor and intracellular signaling proteins [153]. Similar studies in patients with pancreatic cancer have not been performed, but it is well recognized that glutamine is an important fuel (nitrogen donor in nucleotide and amino acid biosynthesis) for the growth of tumor cells. Other studies suggested that inhibiting LPS-induced TLR4 signaling (e.g., by Eritoran/ E5564, TLR4 small molecule antagonist) could improve therapeutic outcomes by preventing cancer metastasis during the perioperative period of colorectal cancer resection [154]. Using a unique ability of pancreatic cells to accumulate omega-3 fatty acids, EPA and DHA were found to induce ROS accumulation and caspase-8-dependent cell death in human pancreatic cancer cells (MIA-PaCa-2 and Capan-2) in vitro [155]. Previous studies demonstrated that EPA inhibited the proliferation of all human pancreatic cancer cell lines in a dose-dependent fashion, and apoptosis was found to be associated with the activation of caspase-3 and suppression of cyclo-oxygenase-2 expression [156]. Polyunsaturated fatty acids (PUFAs) induced apoptosis also in normal gastric and gastric carcinoma cells [157]. Most studies showed that PUFAs caused selective cytotoxicity towards cancer cells via apoptosis, little or no toxicity to normal cells and potentially increased the sensitivity of tumor cells to conventional therapies [158]. A promising phase III trial demonstrated that intravenous ω-3 FAs in combination with gemcitabine improved the activity and quality of life (QOL) in patients with advanced pancreatic cancer [159]. Moreover, the TLR4 protein and mRNA level were markedly down-regulated by PUFA 1 : 1 compared with SFA, however, rats fed the PUFA 1 : 4 diet failed to exhibit these changes [160]. These data highlight the role of the PUFA 1 : 1 diet in the prevention of obesity and related metabolic disorders by suppressing the activation of TLR4, a critical modulator of pro-inflammatory cytokines. An additional nutritional therapy reducing the
expression of TLR4 on tumor cells would probably also inhibit its growth and local inflammation in patients with pancreatic cancer, but these mechanisms are not fully understood. A better known mechanism of omega-3 fatty acid action applies to their pro-apoptotic and anti-inflammatory activity on tumor cells (e.g., regulation of Bcl-2, Bax and c-Myc, and effects on lipids metabolism) [161–163]. Tissue samples derived from patients with head and neck, esophageal, colorectal, liver, pancreatic, skin, breast, ovarian, cervical, and breast cancer were shown to express increased amounts of TLR4 [164], indicating that modulation of the TLR4 signaling pathway would have a beneficial effect on most common cancers.

Apart from TLRs, a significant role in the recognition of pathogen-related molecular patterns and regulation of the innate antibacterial response is also played by other proteins belonging to the pathogen-associated molecular pattern (PAMP) receptors, including peptidoglycan recognition proteins, cytosolic NOD (nucleotide-binding oligomerization domain) proteins and TREM1 receptor (triggering receptor expressed on myeloid cells 1) [165]. Both TLR and NLR pathways are mediated through different adap-

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**Fig. 1.** Schematic diagram of TLR4, MyD88 and TRAF6 down-regulation following glutamine administration in LPS-stimulated peripheral blood leukocytes of pancreatic cancer patients. N-3 PUFAs inhibition of TLR signaling pathway at the extracellular (DHA interfere with TLR4 receptor) and intracellular level: inhibition of the phosphorylation and degradation of the IκB, inhibition of the NF-κB activation and inflammatory cytokines production.

**TLR** – Toll-like receptor; **TIR** – Toll-IL-1 receptor; **TIRAP** – TIR-domain-containing adapter protein; **TRAM** – TRIF-related adapter molecule; **TRIF** – Toll/IL-1 receptor domain-containing adapter inducing IFN; **MyD88** – myeloid differentiation factor 88; **TRAF6** – TNF-α receptor-associated factor 6; **IKK** – inhibitory κB kinases complex; **NF-κB** – nuclear factor κB; **TNF-α** – tumor necrosis factor α; **IL-1** – interleukin 1; **IL-6** – interleukin 6; **LPS** – lipopolysaccharide
NOD1: TNF-α

signaling of the expression of cytokine-coding genes (for of TLRs, which also results in NF-κB

tidoglycan; they can also transmit a signal independently of TLRs, which also results in NF-κB activation and stimulation of the expression of cytokine-coding genes (for NOD1: TNF-α and IL-6, and for NOD2: TNF-α and IL-1β) and adhesive molecules [167]. NOD1 recognizes the compounds (meso-diaminopimelic acid with peptidoglycan) of Gram-negative and some Gram-positive bacteria [168], whereas muramyl dipeptide is the ligand for NOD2, derived from the wall of Gram-positive and Gram-negative bacteria [169, 170]. NOD-like signaling is involved in the regulation of immune homeostasis in the gut. NOD2 deficiency in mice could lead to a compromised epithelial barrier and impaired immune response to commensal microbiota [171]. In turn, NOD1 deficiency resulted in increased tumor formation associated with the inflammation of the colon in mice [172]. NOD1 and NOD2 are involved in the pathogenesis of diabetes, presumably through the recognition of the gut microbiota [173]. It was reported that a NOD2 gene mutation might be also a predisposing factor for colorectal cancer characterized by a higher average age of disease onset in subjects who do not harbor any other genetic predisposition to the disease [174]. Polymorphisms in NOD1/CARD4 and NOD2/CARD15 genes may shift the balance between pro- and anti-inflammatory cytokines, thereby modulating the risk of infection, chronic inflammation and cancer [175]. NOD1/CARD4 and NOD2/CARD15 gene polymorphisms may be associated with altered risk of gastric, colorectal, breast, ovarian, prostate, testicular, lung, laryngeal, liver, gallbladder, biliary tract, pancreatic, small bowel, kidney, urinary bladder and skin cancer as well as non-thyroid endocrine tumors, lymphoma and leukemia. In another meta-analysis, NOD2 rs2066844 C/T, rs2066845 C/G and rs2066847 (3020insC) polymorphisms were potentially associated with an increased cancer risk; moreover, in the cancer type subgroup analysis, the insC/insC+insC/C2 genotype was significantly associated with an increased risk of colorectal cancer, gastric cancer, MALT lymphoma, breast cancer, lung cancer and laryngeal cancer, but not with urogenital cancer, pancreatic cancer, melanoma and non-Hodgkin lymphoma [176]. Furthermore, signaling pathway analyses in a genome-wide association study (data from 3,851 pancreatic cancer cases) revealed that the NOD1 gene was associated with the risk of pancreatic cancer (H. pylori – cytokine signaling pathway) [177]. Further, large-scale and well-designed studies are still needed to confirm the relationship between the NOD gene expression and the risk of pancreatic cancer.

Conclusions and future directions

The results of the studies on the various markers in peripheral blood are strongly affected, i.a., by local disorders of the innate and acquired immune response. Increased expression of biomarkers in peripheral blood reflects a variety of metabolic/immune/genetic disorders associated with both host and tumor defence reactions. These disorders are conducive to the “escape” of cancer cells out of control of the immune system, they inhibit the response to cancer cells and enhance the status of tolerance/immunosuppression. Of significant importance in this process is simultaneously increasing lack of energy substrates, hypoxia of the pancreas tumor tissue caused by poor vascularization and increasing tumor activity accompanied by changes in the number and activity of tumor-infiltrating cell. Most frequently, local change of immunity includes: lymphocyte/neutrophil ratio, changes in subpopulations of T cells (Treg, Th1/Th2, CD8+, CD45RO+ density), dendritic cells, myeloid suppressor cells (MDSC), M1/M2 macrophages, growth factors (e.g., EGF, FGF, VEGF) or cytokines (e.g. IL-5, 6, 10, 13); these changes are not specific to pancreatic cancer, as they are also found in other gastrointestinal tract cancers [178–181]. In pancreatic cancer patients, local cellular immunity disorders (e.g., high or low lymphocyte density in cancer infiltration) have a significant impact on survival [178]. On the other hand, studies of cellular immunity disorders in the peripheral blood showed that MDSCs and Tregs were significantly significantly elevated in pancreatic, esophageal and gastric cancer compared with controls. Moreover, elevated MDSC percentage was associated with an increased risk of death, while in a multivariate analysis, MDSC level was an independent prognostic factor for survival [179]. These studies highlighted basic mechanisms inhibiting or promoting tumor growth, with particular emphasis on their practical diagnostic and therapeutic significance.

As demonstrated by the presented works, in order to increase the effectiveness of methods for early detection of disorders leading to the development of pancreatic cancer, first of all, the knowledge of the local mechanisms regulating signaling pathways associated with innate antibacterial response should be broadened. It should be emphasized that disorders of innate antibacterial response are of fundamental importance in the development of gastrointestinal cancers, including pancreatic cancer. In interpreting the results of disorders of the innate antibacterial immunity mechanisms, the nutritional status of patients and peripheral cellular immunity disorders (e.g., lymphocyte-related apoptosis) should also be taken into account. Previously cited research results indicate that the number of lymphocytes in tumor infiltration (especially the density of tumor infiltrating effector cells) is reduced compared to neutrophils, which is of important prognostic significance. In most studies, the problem of nutritional status disorders of patients (lack of nutritional substances for the proper
functioning of the immune system) is omitted, although it is known that malnutrition is an inherent factor associated with gastrointestinal tract cancers worsening the prognosis. Nutritional deficiencies should be compensated in these patients by appropriate nutritional therapy, which may have a beneficial effect on changing the tumor environment and cancer cells’ metabolism. Metabolic reprogramming is linked to the activation of oncoenes but also to the inhibition of tumor suppressor genes [182]. Interestingly, human pancreatic ductal adenocarcinoma cells rely on a different metabolic pathway than normal pancreatic cells, and, for example in cancer cells, glutamine-derived aspartate is transported into the cytoplasm where it can be converted into oxaloacetate by aspartate transaminase [183]. Furthermore, these studies show that the reprogramming of glutamine metabolism is mediated by oncogenic KRAS, the signature genetic alteration in PDAC, through the transcriptional upregulation and repression of key metabolic enzymes in this pathway. In a recent study, targeting the glutamine metabolism sensitized pancreatic cancer to the PARP-driven metabolic catastrophe induced by β-lapachone, which is an NADPH: quinone oxidoreductase (NQO1)-bioactivatable drug that leads to NADPH depletion (NQO1 expression is highly activated by mutant KRAS signaling) [184]. It should also be noted that in comparison to other cells, pancreatic cancer cells have a unique property of polyunsaturated fatty acid (omega-3) accumulation, which allows to induce apoptosis in them [185]. Additionally, these data also suggest that treating pancreatic cancer cells with omega-3 fatty acid alone, or in combination with gemcitabine, result in an increased inhibition of proliferation, and the mechanism of this inhibition is related to apoptosis progression.

Dysregulation of signaling pathways associated with innate antibacterial immunity is important factor playing a crucial role in pathogenesis, development, prognosis and therapy of pancreatic cancer. The increased interest in this line of research stems mainly from an elevated expression of TLRs and signaling pathway proteins associated with these receptors in pancreatic cancer cells when compared with healthy pancreatic gland. The most interesting are multi-regulatory properties of the TLR receptors (e.g., regulation of proliferation, the activity of NF-kB, gene transcription of apoptosis proteins, regulation of angiogenesis, HIF-1α protein expression) used in experimental studies to better understand the pathogenesis of pancreatic cancer (assessment of bacterial factor participation) and the possibility of therapeutic intervention. This is supported by a number of examples of agonists discussed here (e.g., TLR2/5 ligands, TLR6, TLR9) exhibiting antitumor activity, although parallel stimulation of other TLRs (TLR7/TLR8) produced the opposite effect, while reducing the sensitivity of the tumor to chemotherapy. On the other hand, it appeared that blocking selected TLRs (TLR4, TLR7) could inhibit the development of mouse and human pancreatic cancer cells, while inhibiting the MyD88 signaling pathway in dendritic tumor-infiltrating cells paradoxically stimulated the development of pancreatic cancer. The direction of these studies is promising, but the small number of them does not allow for an accurate assessment of the impact of expression disorders of TLRs, proteins of these signaling pathways, or attempts to block or stimulate them, on the results of treatment of pancreatic cancer patients. It is still difficult to determine whether these disorders may precede the development of pancreatic cancer or whether the discussed parameters can be used as early markers. It is known, however, that the expression disorders of proteins of innate antibacterial response signaling pathways occur not only in tumor tissue but also in peripheral blood leukocytes of pancreatic cancer patients (e.g., increased expression of TLR4, NOD1, TRAF6), which is one of the most important factors facilitating further tumor development.

New hopes for the improvement of pancreatic cancer treatment are associated with genetic testing of microRNA expression changes, the aim of which is to better understand the pathogenesis as well as diagnostic and therapeutic possibilities. MicroRNAs are small non-coding RNAs (19-24 nucleotides long) that regulate gene expression by affecting translation of messenger RNA. MicroRNAs are aberrantly expressed in many tumors and elicit oncogenic or tumor-suppressive functions. Importantly for cancer diagnosis and better understanding of their pathogenesis, altered expression of microRNAs can be determined in the serum and tumor tissues, including paraffin sections. Similarly to investigating cell infiltrations of pancreatic tumor, the assessment of changes in the expression of microRNAs can be a valuable complement to the routine tests (e.g., immunohistochemistry). MicroRNAs mediate signaling network of apoptosis pathways and down-regulation of mutationally activated oncogenic KRAS and its downstream signaling pathways in PDAC [186]. A large body of evidence has revealed dysregulation of miRNAs in PDAC, IPMN and PanIN lesions, suggesting their use as biomarkers for early diagnosis of pancreatic cancer.

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