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REVIEW

Intervention on toll-like receptors in pancreatic cancer

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Abstract

Pancreatic ductal adenocarcinoma (PDA) is a devastating disease with pronounced morbidity and a high mortality rate. Currently available treatments lack convincing cost-efficiency determinations and are in most cases not associated with relevant success rate. Experimental stimulation of the immune system in murine PDA models has revealed some promising results. Toll-like receptors (TLRs) are pillars of the immune system that have been linked to several forms of malignancy, including lung, breast and colon cancer. In humans, TLRs are expressed in the pancreatic cancer tissue and in several cancer cell lines, whereas they are not expressed in the normal pancreas. In the present review, we explore the current knowledge concerning the role of different TLRs associated to PDA. Even if almost all known TLRs are expressed in the pancreatic cancer microenvironment, there are only five TLRs suggested as possible therapeutic targets. Most data points at TLR2 and TLR9 as effective tumor markers and agonists could potentially be used as *e.g.* future adjuvant therapies. The elucidation of the role of TLR3 in PDA is only in its initial phase. The inhibition/blockage of TLR4-related pathways has shown some promising effects, but there are still many steps left before TLR4 inhibitors can be considered as possible therapeutic agents. Finally, TLR7 antagonists seem to be potential candidates for therapy. Independent of their potential in immunotherapies, all existing data indicate that TLRs are strongly involved in the pathophysiology and development of PDA.

Key words: Pancreatic cancer; Pathophysiological mechanism; Toll-like receptor; Intervention; Adjuvant therapy

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Core tip: The combination of high mortality rates and a tremendously complex pathophysiology makes pancreatic ductal adenocarcinoma (PDA) an enormous challenge. We summarize the current knowledge about the importance of toll-like receptors (TLR) in PDA. Since both tumor and tumor-related cells express TLRs, intervention on TLR-related pathways may represent future candidates for therapy.

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INTRODUCTION

Disorders of the pancreas are leading causes of morbidity and mortality. Despite advanced surgical and/or oncological treatment strategies, pancreatic ductal adenocarcinoma (PDA) is still associated with an extremely poor prognosis with a median survival of 6 mo and a 5-year survival rate less than 1%-2%[1,2]. PDA represents the fourth cause of cancer-related deaths and its incidence is rising in most countries[3].

The causes of PDA are mainly unknown. A family history is found in up to 5%-10% of patients[4]. Known

risk factors for PDA are among others tobacco smoking, diabetes mellitus, obesity and chronic pancreatitis[5-7]. Pancreatic intraepithelial neoplasia (PanIN) in the ductal epithelium has been suggested as the primordial precursor of PDA[8]. As PanIN progress to carcinoma, accumulated mutations might result in the activation of the *KRAS2* oncogene, loss of CDKN2A/p16 and/or the inactivation of TP53 and SMAD4[9]. Likewise, stellate cells are major players in PDA, as they are fundamental for the development of the characteristic desmoplastic stroma found in PDA[10]. Pancreatic cancer stem cells might be important in treatment resistance and metastasis. A large range of cell populations, such as tumor-associated macrophages (TAMs), have been reported as central in PDA[11,12]. The current knowledge of the pathophysiology of PDA has elegantly been summarized by Hidalgo[13,14].

At the time of diagnosis, most patients have already developed locally advanced (stages Ⅱ or Ⅲ) or metastatic (stage Ⅳ) disease and palliative treatment is the only alternative. Gemcitabine is a nucleoside analogue with a broad-spectrum against solid tumors that for long has been used as first-line treatment. In PDA, gemcitabine increases the quality of life of many patients, but merely prolongs the mean survival by one month[15]. Furthermore, a majority of patients do not respond to gemcitabine due to lack of the necessary nucleoside transporter, and the total costs and side-effects related to gemcitabine overtreatment are high[16,17]. FOLFIRINOX (5FU/leucovorin, irinotecan and oxaliplatin) is currently a first-line treatment for metastatic PDA as the regime is more active than gemcitabine at overall survival, progression-free survival and response rate. Moreover, the degradation of the quality of life is also delayed by FOLFIRINOX[18]. However, the regime is more expensive than gemcitabine and not suitable for all patients due to its toxicity. Hence, in most developing countries, gemcitabine is still the gold standard. Thus, current chemotherapeutic strategies lack proper cost-efficiency determinations and are not effective in the vast majority of cases.

In order to increase survival rates in PDA, it is imperative to find novel therapies that specifically target tumor cells and/or associated cell populations and stroma. Toll-like receptors (TLRs) are pillars of the immune system that have been linked to major cancer forms, including lung, breast and colon cancer[19-21]. In humans, TLRs are expressed in the pancreatic cancer tissue and in several cancer cell lines, whereas they are not expressed in the normal pancreas[22,23] (Table 1). TLRs thus appear to play a role in the pathophysiology of PDA (Table 2, Figure 1) and may thereby also represent targets for intervention (Table 3). In the present review, we explore the current knowledge concerning the role of different TLRs associated to PDA.

TOLL-LIKE RECEPTORS

TLRs are pattern recognition receptors that recognize numerous pathogen-associated molecular patterns (PAMPs) derived from virus, pathogenic bacteria, pathogenic fungus and parasitic protozoa. Likewise, TLRs can start immunological reactions against endogenous molecules released into the extracellular compartment under *e.g.*, stress or tissue damage[24]. TLRs are type I integral membrane glycoproteins expressed in various cell compartments, and in humans the expression of ten different TLRs (TLR1 to TLR10) has been reported[25]. Upon activation, TLRs form heterodimers or homodimers, and an activating signal is started. After the recruitment of adaptor molecules, TLRs can activate two major intracellular signaling pathways. All TLRs, except TLR3, can activate a MyD88-dependent pathway, causing the transcription of pro-inflammatory genes through the activation of nuclear factor κβ (NFκB) and/or the activation of activating protein 1[24,26]. An alternative, non MyD88-dependent pathway, can be initiated by TLR3 and TLR4. In the TRIF-pathway, the activation of interferon-regulated factors (IRF) *via* TRIF results in the synthesis of interferon (IFN) and/or the activation of NFκB[24].

TLR2-PROMISING ADJUVANT THERAPY

Mainly expressed on the plasma membrane, TLR2 is found in a large diversity of cells of the immune system[27]. In addition to its role in infectious diseases, TLR2 has been associated to *e.g.*, atherosclerosis, asthma and renal disease[28-30].

Macrophage activating lipopeptide-2 (MALP-2) is a synthetic lipopeptide that activates immune responses through TLR2 and TLR6[31,32]. In syngeneic subcutaneous and in orthotopic murine models the local administration of MALP-2 results in significant tumor growth reduction and prolonged survival[33]. Furthermore, the MALP-2 anti-tumor effect is enhanced by co-treatment with gemcitabine. However, the metastatic potential of cancer cells is not reduced by MALP-2 administration. MALP-2 might exert its effects through CD8+ lymphocytes and NK-cells since the murine Panc-2 cell line used for this experiment do not express TLR2. Hypothetically, MALP-2 activates dendritic cells (DCs) in a TLR2/TLR6-dependent manner[34]. A subsequent phase Ⅰ / Ⅱ trial showed promising results[35]. Ten patients in different PDA disease stages were included, both with "radical" surgery or palliative procedures leaving the pancreatic tumor behind. MALP-2 was injected intratumorally during surgery and six patients received adjuvant chemotherapy. The drug was well tolerated and a mean survival of 17.1 mo was observed. The median survival was 9.3 mo and no metastases were reported during follow-up. Despite the limited number of patients, the reported mean survival was remarkably high. The local administration of MALP-2 appears to upregulate the activation of both the innate and the adaptive immune system, resulting in decreased tumor proliferation and metastasis. Still, it is unclear if MALP-2 has a future as adjuvant therapy in PDA, since no further trials have been reported up to date. In addition, several less expensive TLR2 agonists appear to have similar biochemical properties when compared to

Table 1 Toll-like receptors found in human pancreatic adenocarcinoma cell lines

Cell line	Source	Phenotype	Expressed TLR	Ref.
AsPC-1	Metastasis: ascites	Du	TLR3, TLR4, TLR9	[23,57,58]
BxPC-3	Primary tumor	Du	TLR2-4	[40,50,57]
CFPAC	Metastasis: liver	Du	TLR4	[57]
Colo357	Metastasis: lymph node	Un	TLR3, TLR7	[48]
GER	Primary tumor	An	TLR9	[72]
MIA PaCa-2	Primary tumor	An	TLR2-4, TLR7, TLR9	[23,40]
MDAPanc-28	Primary tumor	Du/Ac	TLR2-4, TLR7, TLR9	[23]
Panc-1	Primary tumor	Du/An	TLR2-4, TLR7, TLR9	[23,40,50,58,79]
Panc-89	Metastasis: lymph node	Du	TLR3 , TLR7	[48]
PancTu-1	Primary tumor	Du	TLR3, TLR7	[48]
Pt45P1	Primary tumor	Du	TLR3, TLR7	[48]
SU.8686	Metastasis: liver	Du	TLR2	[41]
SW-1990	Metastasis: spleen	Du	TLR2-4, TLR7, TLR9	[23]
T3M4	Metastasis: lymph node	Du	TLR9	[77]

TLR: Toll-like receptor; Du: Ductal; Ac: Acinar; An: Anaplastic; Un: Undefined.

Table 2 Toll-like receptors expressed in pancreatic ductal adenocarcinoma and their reported implications

	Pathophysiological significance	Ref.
TLR2	Cell growth	[33,40,43]
	Immunosuppression	[33,41,43]
	Mean survival	[33,35]
	Progression and metastasis	[43]
TLR3	Carcinogenesis	[47]
	Cell growth and migration	[50]
	Immune responses	[48]
TLR4	Angiogenesis	[63]
	Carcinogenesis	[49]
	Cell growth	[49,57,61]
	Epithelial-to-mesenchymal transition	[61]
	Leukocyte recruitment and genomic instability	[57]
	Mean survival	[62]
	Progression and metastasis	[49,58,61]
	Stromal expansion	[49,61]
TLR7	Carcinogenesis, stromal expansion, progression and metastasis	[67]
	Immune responses	[48]
TLR9	Cell growth	[77,79]
	Mean survival	[77]
	Metastasis	[72,77,79]

TLR: Toll-like receptor.

MALP-2.

Protein-bound polysaccharide-K (PSK, Krestin®) is a natural remedy derived from highly purified mushroom extracts (*Trametes versicolor*) that since decades has been used as adjuvant therapy in cancer[36]. Even if the mechanisms are only partially known, PSK is thought to be a novel TLR2 agonist and it has documented therapeutic effects in colorectal and lung cancer[37-39]. Moreover, PSK promotes apoptosis and inhibit tumor growth in various human PDA (hPDA) cell lines[40]. Even if PSK-related cancer cell apoptosis is unlikely to be mediated through TLR2, the inhibition of the later significantly reduced the positive effects of PSK in all cell lines challenged. Thus, TLR2-pathways might be (if only in part) involved in the tumor suppressor effect of PSK.

TLR2 is also a promising cell-surface target since its protein expression is specifically increased in hPDA tissue[22]. Designed, fully synthetic high affinity TLR2 agonists have been studied with encouraging outcome. Derived from natural TLR2 ligands and also from MALP-2, these new compounds are able to induce the immune system when given as vaccine adjuvants in murine PDA (mPDA) models[41]. These results imply a potential in developing high affinity tumor targeted therapies through TLR2. A particularly potent compound has been conjugated with a near-infrared fluorescent dye, the novel Dmt-Tic-Cy5. The combination of Dmt-Tic-Cy5 and 3D imaging methods was applied in the intraoperative detection of tumor masses in a mouse xenograft model[42]. Using Dmt-Tic-Cy5 as a tumor marker during surgery in mice, successful R0 resections were obtained. Future applications of this technic could include the detection of early tumors or the improvement of current surgical procedures in hPDA.

Pancreatic adenocarcinoma upregulated factor (PAUF) is a protein overexpressed in hPDA and other types of cancer[43]. PAUF appears to modulate the metastatic potential of cancer cells and it upregulates the expression of CXCR4, the later being related to increased cancer cell motility[44]. PAUF induce the expression of the cytokines RANTES and MIF *via* TLR2 and it is also associated with the inhibition of CXCR4-dependent and TLR2-mediated NFκB activation, with subsequent decreased tumor necrosis factor-α levels[45]. Theoretically, PAUF might contribute to tumor persistence *via* the disruption of TLR2-dependent anti-tumor pathways in cancer.

In summary, TLR2 is not only expressed in tumor tissue but also in several hPDA cell lines (Table 1). Since TLR2 is present in both primary tumor cell lines and in cell lines from metastases, the receptor may be a novel target for immunotherapy in hPDA. The clinical significance of TLR2-targeting can become important in the future since the marker is present in up to 70% of resected tumors[22] but mainly absent in the normal pancreas. While the pathophysiological role of TLR2 in mPDA seems to be complex (Table 2, Figure 1), TLR2 agonists have shown promising results in animal models and in a phase I / II clinical trial (Table 3).

TLR3-UNEXPLORED IMPLICATIONS

TLR3 is a nucleic acid-recognizing receptor expressed as dimers on endosomal membranes of DCs and mono-

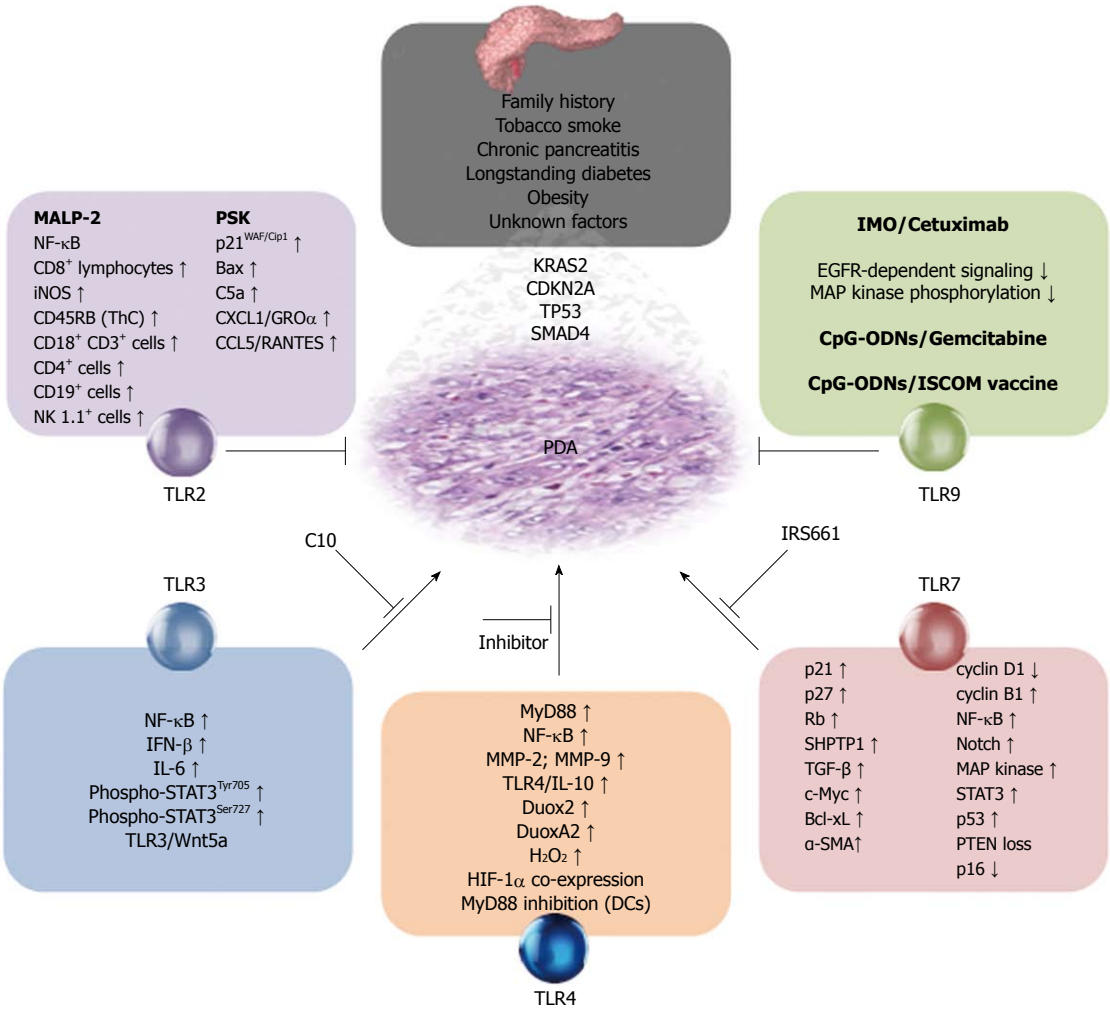

Figure 1 Toll-like receptors in the pathophysiology pancreatic ductal adenocarcinoma. TLR: Toll-like receptor; MALP-2: Macrophage activating lipopeptide-2; PSK: Polysaccharide-K; C10: Phenylmethimazole; IRS661: Immunoregulatory sequence 661; CpG-ODN: CpG oligodeoxynucleotide; IMO: Immunomodulatory nucleotides; TGF-β: Transforming growth factor-β; α-SMA: α-smooth-muscle antibody. NF-κB: The nuclear factor NF-κB.

cytes. Besides its role in viral infections, TLR3 has been linked to chronic pancreatitis and breast cancer[46,47].

Polycytidylic acid (Poly I:C) is a well-known TLR3 agonist capable of inducing cell lysis in hPDA cell lines by enhancing the cytotoxic activity of γδ T cells *in vitro*[48]. However, Poly I:C has also been reported to accelerate pancreatic carcinogenesis in KRAS-mutated mice[49].

TLR3 expression in hPDA cell lines is correlated with increased tumor cell growth and constitutive Wnt5a expression[50]. Wnt-associated pathways are related to a vast variety of cellular processes in embryogenesis and carcinogenesis[51]. Phenylmethimazole (C10) is a TLR3 inhibitor able to suppress the dsRNA induced, TLR3-mediated IRF3/IFN-pathway, independent of Wnt5a. The administration of C10 leads to less tumor development in a xenograft murine model. Importantly, C10 decreased TLR3 expression and significantly inhibited hPDA cell growth and motility/migration. The expression of TLR3 in tumor cells might result in increased interleukin (IL)-6 levels[52]. C10 effects could then be mediated by the inhibition of phosphorylated STAT3 *via* the disruption of TLR3/Wnt5a-related pro-inflammatory IL-6 expression in hPDA.

Even if TLR3 is constitutively expressed in primary hPDA cell lines (Table 1), it is unclear which role TLR3 plays in hPDA. Opposite results have been reported when TLR3 inhibitors have been tried. Hence, no conclusions can be made at this point.

TLR4-IS INHIBITION THE ANSWER?

Being the first TLR identified, TLR4 is widely expressed as homodimers or heterodimers with TLR6 on the plasma membranes of many immune cells. TLR4 has been linked to several diseases, including obesity, acute pancreatitis and breast cancer[18,53,54].

TLR4 is overexpressed both in mPDA and hPDA[49]. Stromal leukocytes from patients have increased TLR4 expression. Interestingly, the upregulation is also found both in epithelial and stromal cells in KRAS-mutated mice. Moreover, TLR4-inhibition in these mice had protective effects against tumorigenesis and TLR4$^{-/-}$ animals had a slower tumor growth. However, the inhibition of MyD88-dependent and TRIF-pathways had opposite

Table 3 Toll-like receptors and their intervention in pancreatic ductal adenocarcinoma

	Substance/compound	Intervention	Effects	Ref.
TLR2	MALP-2 (G)	Activation	Induce lymphocyte invasion and tumor necrosis	[33]
			Inhibit tumor growth	[33]
			Prolongs mean survival	[35]
			Reverse tumor-associated immunosuppression	[33]
	Polysaccharide-K (G)	Activation	Inhibit tumor growth and induce apoptosis in tumor cells	[40]
	Dmt-Tic-Cy5	Activation	Acts as vaccine adjuvant in pancreatic cancer	[41]
			Target imaging and therapy	[41,42]
	PAUF	Mixed	Facilitates tumor growth	[43]
			Promotes tumor immune-resistance	[43]
TLR3	Polycytidylic acid	Activation	Accelerates carcinogenesis	[49]
			Induces T cell invasion and tumor lysis	[48]
	Phenylmethimazole	Inhibition	Inhibits tumor growth and migration	[50]
TLR4	Lipopolysaccharide	Activation	Accelerates carcinogenesis	[49]
			Induce desmoplastic stroma	[49]
			Induce increased H_2O_2 extracellular production	[57]
			Increased invasiveness	[58,61]
			Induce M2-polarization in tumor-associated macrophages	[61]
TLR7	Imiquimod	Activation	Induce T cell invasion and tumor lysis	[48]
	IRS661	Inhibition	Prevent tumor progression and stromal expansion	[67]
			Regulates cell cycle in cancer cells	[67]
TLR9	CpG-ODN 1816/26 (G')	Activation	Delays tumor development, reduce invasiveness	[72]
			Prolongs mean survival	[72]
	IMO (C)	Activation	Prolongs mean survival, inhibit tumor growth and migration	[77]
			Reestablish cetuximab sensibility in cancer cells	[77]
	CpG-ODN 2216	Activation	Inhibits tumor growth and migration	[79]

TLR: Toll-like receptor; MALP-2: Macrophage activating lipopeptide-2; PAUF: Pancreatic adenocarcinoma upregulated factor; IRS661: Immunoregulatory sequence 661; CpG-ODN: CpG oligodeoxynucleotide; IMO: Immunomodulatory nucleotides. (G): Synergism when combined with gemcitabine; (G'): Effect mainly when combined with gemcitabine; (C): Effect merely when combined with cetuximab.

effects in mPDA. While MyD88-inhibition clearly accelerated tumor development and gave rise to highly aggressive TP53 mutated cancer cells, TRIF-inhibition had antitumor effects. MyD88-inhibition could induce aggressive cancer cells even in TRIF-deficiency co-existence.

Even if MyD88 blockage has been associated with a decreased tumor development in other cancer forms[55], the presence of DCs in PDA microenvironment appears to be the main factor for MyD88-dependent tumor-stimulating effects. Upon MyD88 blockage, DCs seem to induce pancreatic antigen-restricted Th2-deviated $CD4^+$ T cells[49]. Furthermore, the abundance of Th2 cells in hPDA is linked to a worsened prognosis[56].

Inflammatory cytokines can induce NFκB activation in mPDA. LPS and INF-γ challenge results in increased production of extracellular H_2O_2 in primary hPDA cell lines[57]. Through TLR4, the activation of NFκB might enhance the transcription of dual oxidase 2, trigger leukocyte recruitment and genetic instability. In hPDA cell lines, LPS challenge induced improved invasiveness *via* TLR4/MyD88-depending pathways[58]. Moreover, RNAi silencing TLR4 or MyD88 completely reversed the effects of LPS. NFκB activation might induce increased expression of matrix metalloproteinases (MMPs) in mPDA. MMP-2 and MMP-9 overexpression is related to the progression of hPDA, and its blockage has been subject of intensive research[59]. Thus, LPS may act through a TLR4-MyD88-NFκB axis that finally leads to MMP-9 overexpression and thereby to increased invasiveness *in vitro*[60].

The overexpression of MMPs has also been coupled to TAMs. M2-polarized TAMs mediate EMT, induce cancer cell proliferation and migration in hPDA cells *in vitro*[61]. These effects may partially be achieved through TLR4. TLR4 overexpression in M2-polarized macrophages could lead to IL-10 release with impact on the EMT and thereby on the metastatic potential of the cancer cells.

hPDA is characterized by a poor vascularization. Thus, the role of angiogenesis in hPDA remains controversial[9]. In humans, hypoxia-inducible transcription factor alpha (HIF-α) is overexpressed in resected pancreatic cancer tissue. Moreover, a positive correlation between mRNA/protein HIF-α levels and mRNA/protein TLR4 levels in primary tumors and metastases has been found. TLR4 was expressed in 69.2% of the analyzed tumor tissue. Besides, the expression of either TLR4 or HIF-α was related to a decreased survival rate and when both were expressed, an accumulative effect was observed[62]. Some data imply that hypoxia in solid tumors, such as hPDA, induces HIF-α overexpression, which might be responsible for the expression of TLR4 in hPDA cells *in vitro* and in a xenograft murine model[63]. Here, TLR4 was found in 76 % of the tumor tissue but no data on average survival or prognosis was presented.

The inhibition/blockage of TLR4-related pathways has shown some promising results, but there are still many steps left before TLR4 inhibitors can be considered as possible therapeutic agents. Since both stromal cells and primary tumor cells express TLR4, it is plausible

that TLR4 ligands found in the inflammatory tumor microenvironment initiate complex interactions between the different cell populations. This might in turn lead to the secretion of tumor stimulating cytokines and the recruitment of further cell populations into the tumor stroma. Since hypoxia and TLR4 ligands are common in the tumor stroma, the upregulation of TLR4 and HIF-α in hPDA could be auto-stimulatory. Poor prognosis can then be partially predicted, as a highly hypoxic tumor stroma is less sensitive for radiotherapy and disrupt the delivery of chemical agents into the primary cancer cells.

TLR7-PROMOTING CANCER PROGRESS

TLR7 is a nucleic acid-recognizing receptor expressed as dimers on endosomal membranes of APCs and leukocytes. TLR7 activation is currently used for the treatment of various malignancies, such as melanoma and breast cancer[64]. Like TLR3, TLR7 has also been used to enhance cytotoxic activity in γδ T cells *in vitro*[48].

The role of TLR7 in mPDA has been reported previously[65,66]. Upregulated TLR7 is found in epithelial cells and macrophages, DCs, neutrophils, and B- and T-cells of the tumor microenvironment. In hPDA, the expression of TLR7 is increased both in epithelial ductal cells and inflammatory cells within the tumor stroma.

Moreover, the administration of ssRNA40, a TLR7 agonist, results in pronounced tumor growth and stromal expansion in mice. In KRAS-mutated mice, the tumor-stimulating effects of TLR7 appears to be mediated by a complex array of events, including loss of expression of PTEN, p16 and cyclin D1 and upregulation of among others p21, p27, p53, c-Myc, SHPTP1, TGF-β, PPARγ and cyclin B1. Moreover, ssRNA40 challenge resulted in the activation of STAT3, MAP kinase, Notch and NFκB pathways. Notch target genes were downregulated, giving rise to the hypothesis that Notch, together with NFκB, might mediate inflammation in the tumor microenvironment, thus promoting tumor persistence and metastatic potential[67].

Importantly, TLR7 stimulation is not self-sufficient for malignant transition when KRAS mutations are absent. Equally important, mice with $TLR7^{-/-}$ phenotype seem to be protected against tumor progression. The administration of IRS661, an oligonucleotide inhibitor of TLR7, prevented tumor progression and stromal expansion in mice[67]. IRS661 treatment decreased the expression of p21, p27, p-p27, cyclin B1, CDK4 and p-STAT3 in mice with invasive PDA. Thus, TLR7 inhibition was able to affect cell cycle regulation in already formed pancreatic tumors. However, the expression of Rb or TP53 was not affected by IRS661.

The evidence of the importance of TLR7 in mPDA is strong and TLR7 antagonists are without doubt promising experimental adjuvant agents that must be further evaluated. Importantly, PanIN in humans do not express TLR7 with the same intensity as established hPDA tumors. Moreover, the expression of TLR7 appears to increase with tumor progression and it is found in nearly 50% of the advanced tumors[67]. TLR7 may induce tumor progression in a KRAS-dependent manner since the mutation must be present for TLR7-mediated tumor progression in mice. As KRAS2 is mutated in over 90% of hPDA[18], these may only be a minor obstacle for the future clinical use of TLR7-targeting.

TLR9-AGONISTS AS FUTURE ADJUVANT THERAPY?

As TLR3 and TLR7, TLR9 is expressed on endosomal membranes of several immune cells, including macrophages, B cells and DCs[68]. Besides its role in bacterial, viral or malaria infection, TLR9 has been linked to acute pancreatitis and cancer[54,69].

Synthetic TLR9 agonists (CpG-ODNs) are oligodeoxynucleotides containing CpG motifs that have been used as vaccine adjuvants or as antiallergic agents[70]. In combination with vaccines based on immune stimulatory complexes, a TLR9 agonist inhibits the tumor immune evasion in mPDA[71]. It is believed that CpG-ODNs can activate NK-cells, DCs and cytotoxic T cells, thus initiating anti-tumor immune responses. TLR9 is highly expressed in the tumor microenvironment and in circulating leukocytes in a murine xenograft PDA model. CpG-ODNs treated mice had a reduced tumor spread to the diaphragm, liver and spleen and the combination of gemcitabine and CpG-ODNs resulted in delayed development of bulky disease, less metastasis and improved survival, when compared to gemcitabine monotherapy[72].

The epidermal growth factor receptor (EGFR) is overexpressed in 50%-60% of hPDA[73]. Cetuximab is a monoclonal anti-EGFR antibody that has shown promising results experimentally, but not clinically in hPDA[74,75]. Immunomodulatory nucleotides (IMO) are second-generation CpG-ODNs with higher metabolic stability. IMO interferes with EGFR-dependent signaling and has thereby a synergistic effect with anti-EGFR agents[76]. In combination with cetuximab, IMO inhibits cell growth in hPDA and cancer progression in KRAS-mutated murine cell lines[77]. Importantly, in cetuximab-resistant cell lines, IMO potentiated the activity of cetuximab. The administration of IMO resulted in tumor growth inhibition and prolonged survival in a murine xenograft model. The associations between EGFR/TLRs interactions and carcinogenesis are slowly being elucidated. However, the impact on hPDA is still unexplored[78].

Another CpG-ODN (ODN2216) has shown antiproliferative properties in an hPDA cell[79]. Tumor cell growth, replication rate and migration ability were decreased in cells challenged with ODN2216. The effects seem to be time- and dose-dependent. Moreover, the expression of TLR9 is more pronounced in hPDA tissue than in peritumoral ones (73.3% *vs* 33.3%)[79].

As TLR2, TLR9 appears to be a promising tumor marker. Likewise, TLR9 agonists could be used as adjuvant therapy by themselves or in combination with al-

ready established chemotherapies (Table 3). Nonetheless, the pathophysiological role of TLR9 in hPDA is mainly unexplored.

CONCLUSION

The role of the immune system in cancer is an area of intensive research. Cancer cells have the ability to evade immune responses and promote tumor phenotypes and pathways in immune cells. TLRs are related to several cancer forms, and immunotherapies involving TLRs are a reality[27]. At least thirty new clinical trials evaluating TLRs agonist and cancer have started since May 2012[80].

The combination of high mortality rates and a tremendously complex pathophysiology makes PDA an enormous challenge. The role of inflammation and immune cells in PDA cannot be stressed enough[81]. Both MyD88-dependent cascades and TRIF-pathways have been associated with tumor growth, survival and metastatic potential in PDA[65]. Even if almost all known TLRs are expressed in the pancreatic cancer microenvironment, there are only five TLRs suggested as potential therapeutic targets.

Importantly, the effects of TLRs agonists and antagonists in PDA are presumably mediated by the inducement of anti-tumor immune response. This requires access to the primary tumor site. Moreover, TLR-targeting can theoretically disrupt important pathways in primary tumor cells with therapeutic effects. Thus, TLR-based agents must either be administered intratumorally or delivered through the tumor stroma. The recognition of specific tumor targets is then imperative for the application of TLRs intervention in PDA. In clinical practice, CA 19-9 is widely used as hPDA marker. CA 19-9 is a relatively specific marker useful as indicator for advanced disease or tumor recurrence after surgery. However, as pancreatic cancer progress and spreads beyond the pancreas, the accumulation of abnormalities might change the sensitivity and/or specificity of tumor markers since metastases may differ profoundly from the primary tumor[82]. We have recently propose mucin 4 (MUC4) as a novel tumor marker in hPDA. MUC4 is found in both primary and matched metastatic tumors with a high level of concordance (82 %)[83]. Specific tumor markers open the door for efficient drug delivery *via e.g.*, nanotechnology. For instance, targeted liposomal delivery of TLR9 ligands in cancer has already been evaluated with encouraging results[84].

Independently of their potential in immunotherapies, all existing data indicate that TLRs are strongly involved in the pathophysiology of PDA (Figure 1). The role of TLRs in PDA is not limited to the direct effect on tumors or associated cells. TLRs are also involved in the pathophysiology of several risk factors for hPDA, such as chronic pancreatitis, diabetes and obesity[47,85].

The present paper summarizes the current understanding of interventions on TLRs in PDA. Despite initial encouraging results, further research and elucidation of involved mechanisms is demanded.

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