Opisthorchiasis-Induced Cholangiocarcinoma: How Innate Immunity May Cause Cancer

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Abstract

Innate, inflammatory responses towards persistent *Opisthorchis viverrini* (OV) infection are likely to contribute to the development of cholangiocarcinoma (CCA), a liver cancer that is rare in the West but prevalent in Greater Mekong Subregion countries in Southeast Asia. Infection results in the infiltration of innate immune cells into the bile ducts and subsequent activation of inflammatory immune responses that fail to clear OV but instead may damage local tissues within the bile ducts. Not all patients infected with OV develop CCA, and so tumourigenesis may be dependent on multiple factors including the magnitude of the inflammatory response that is activated in infected individuals. The purpose of this review is to summarize how innate immune responses may promote tumourigenesis following OV infection and if such responses can be used to predict CCA onset in OV-infected individuals. It also hypothesizes on the role that *Helicobacterspp*., which are associated with liver fluke infections, may play in activation of the innate the immune system to promote tissue damage and persistent inflammation leading to CCA.

1. INTRODUCTION

1.1 *Opisthorchis viverrini*, An Endemic Disease Throughout Southeast Asia

Infection with the liver fluke *Opisthorchis viverrini* (OV), or opisthorchiasis, has long been linked with the onset of cholangiocarcinoma (CCA). The parasite infects human hosts (and other piscivorous animals) when they eat OV-infected, undercooked/raw cyprinoid fish. Rates of OV infection are especially high in the northeastern region of Thailand (Sithithaworn et al., 2012a; Yeoh et al., 2015), where persistent infection is a consequence of eating raw fish, often as part of traditional dishes such as '*Koi Pla*' and '*Pla Som*'. Numerous control programmes have been implemented in OV endemic areas (Sripa et al., 2015), but despite initial decreases in opisthorchiasis following the introduction of such programmes, rates of CCA remain high in Thailand and neighbouring countries, such as Lao-PDR, Cambodia and Vietnam. This disease therefore continues to place a severe burden on both the health services and the regional economy (Andrews et al., 2008), highlighting an urgent need for an improved understanding of OV pathogenesis and of the processes that lead to the development of CCA. OV infection and liver disease are often asymptomatic, and there is currently no cure for CCA, and often metastasis has occurred before detection, with patients surviving for only short periods (sometimes less than 4 months) post diagnosis (Luvira et al., 2016). To date, the antihelminthic drug

praziquantel can be used as a chemotherapeutic, but this can only treat OV infection, and not CCA itself.

There is considerable heterogeneity in pathological outcomes following OV infection, with some individuals infected for their entire lives without showing any clinical symptoms. However, approximately 25% of those infected with OV develop advanced periductal fibrosis and approximately 1% develop CCA (Mairiang et al., 2012). The reasons for this disease heterogeneity are not fully understood, but it has been hypothesized that a 'proinflammatory phenotype' predisposes particular individuals to develop advanced periductal fibrosis (APF) following OV infection that may lead to CCA (Sripa et al., 2012a). If it is the case that OV-induced CCA is related to patient-specific immune responses, then identifying these responses may make the onset of cancer more predictable or perhaps preventable. To date, whilst many reviews have suggested a possible link between OV-induced CCA and adaptive inflammatory responses, there is a paucity of literature describing specific innate immune cell responses to OV. If the nature of the nonspecific, innate immune responses towards OV can be identified and are confirmed to be responsible for causing or contributing to the development of CCA, these may represent novel targets for treatment and prevention of OV-induced CCA (Sripa et al., 2012a). In addition, these responses may serve as biomarkers that could identify individuals at risk of developing CCA following OV infection and hence enable a more targeted approach to prevention and therapy. This review aims to improve our understanding of the innate immune responses elicited by OV infection and discusses how this may be linked to the onset of CCA. It highlights a possible link between liver fluke infection, *Helicobacter* coinfection and innate immune cell activation in disease pathology.

1.2 *Opisthorchis viverrini*: An Infectious Agent Capable of Driving Cancer

Infection with OV can cause jaundice, cholangitis and CCA, which is a malignancy of the bile duct epithelium, and relatively rare worldwide but highly prevalent in Mekong countries (Sithithaworn et al., 2012b). CCA is usually associated with OV infection, but alcohol, smoking and diets containing nitrogenous compounds may all contribute towards malignancy (Sithithaworn et al., 2014). There are two recognized types of CCA, OV-associated and non-OV-associated, which have differential carcinoma expression profiles, but the 5-year survival rate for both pathologies is <5% (Sripa and Pairojkul, 2008; Ghouri et al., 2015). The initiation of CCA in

opisthorchiasis patients occurs in areas of inflammation, fibrosis and proliferating epithelial cells that may have an increased risk of tumourigenesis when also exposed to carcinogens (Sripa et al., 2007). Due to its association with CCA, OV is recognized as a Group 1 carcinogen and one of few infections that have known carcinogenic properties; others include Hepatitis B and *Helicobacter pylori* infections (IARC, 2011). Other liver flukes endemic in Southeast Asia, including *Clonorchis sinensis* and *Opisthorchis felineus* (Lim, 2011), are less frequently associated with CCA.

There are three hypothesized causes of OV-induced CCA: (1) chronic or persistent inflammatory responses towards persistent opisthorchiasis; (2) anti-apoptotic/immunomodulatory properties of *O. viverrini* excretory/secretory (OVES) products products; and (3) structural damage caused by OV feeding on epithelial cells (Sripa et al., 2007; Sripa et al., 2012a). All three processes may be involved and may contribute to CCA. Inflammatory responses, particularly those that result in the generation of free radicals, may inflict oxidative and nitrogenous damage on the DNA of local cells, a well-established hallmark of cancer (Murata et al., 2012; Yongvanit et al., 2012a). The recent discovery of an association between OV infection with *Helicobacter* infections (*H. pylori* and *H. bilis*) (Deenonpoe et al., 2015, 2017) and the known effects of these bacteria in both cancer pathogenesis and immune cell activation suggest an additional mechanism to explain how inflammation may contribute to the development of CCA (Sripa et al., 2017).

The fact that opisthorchiasis is in decline in some regions, but CCA remains prevalent, suggests that either OV infection alone cannot directly induce CCA, and that a multitude of factors are involved in CCA pathogenesis, or alternatively, that OV infection is an initiator of cancer. Thus, if tissue damage is initiated by OV-induced inflammation, then a vicious cycle of inflammation—tissue damage: inflammation leading to CCA—may exist even when the OV infection has been cleared by treatment (Fig. 1). This concept is supported in hamster models, where CCA only develops when OV infection is combined with exposure to subcarcinogenic dose of nitrosamines such as N-nitrosodimethylamine (NDMA) (Thamavit et al., 1978; Boonmars et al., 2011). Nitrosamines from fermented fish are common in the diet of those who eat traditional, raw fish meals and are also linked with carcinogenesis (Bartsch and Montesano, 1984). Infection with OV alone causes hamsters to develop intrahepatic and extrahepatic biliary damage/abnormalities associated with CCA (Thamavit et al., 1978), but not the onset of cancer itself. It would appear that infected hosts cannot produce an

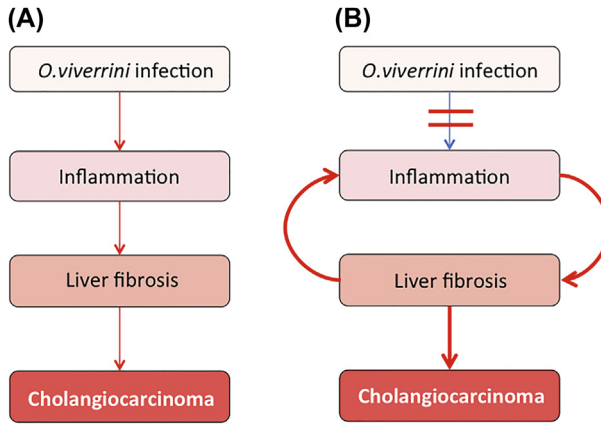

Figure 1 Proposed role of persistent inflammation in the development of cholangiocarcinoma following infection with the liver fluke, *Opisthorchis viverrini* (*O. viverrini*). In (A), infection with the liver fluke leads to inflammation that can (along with other factors, see Fig. 2) result in the development of fibrosis and subsequently cholangiocarcinoma. In (B), it is proposed that, in some individuals, inflammation can persist even after the parasite has been cleared (e.g., by praziquantel treatment). This persistent inflammation may lead to a vicious cycle of inflammation/damage that can lead to fibrosis and ultimately cholangiocarcinoma. In this proposed model, inflammation can drive liver fibrosis, whereas liver fibrosis can also drive further cycles of inflammation.

efficient immune response to clear the parasite and/or that OV is capable of evading or modulating immune responses, which allows its survival (McSorley et al., 2013). The persistent inflammatory responses directed towards OV indirectly promote tumourigenesis by providing the suitable microenvironment for tumours to develop but may require additional carcinogens such as nitrosamines and alcohol, to initiate tumourigenesis (Sripa et al., 2007, Fig. 2). It is feasible that OV infection physically blocks the bile ducts, with the result that the accumulated endogenous nitrosamines and free radicals have prolonged exposure to local cells and attain very high local concentrations, increasing the chances of DNA damage (Sripa et al., 2007).

The hypothesized 'proinflammatory phenotype' may only reveal itself in instances of long-term or repeated infections, such as during OV infection or untreated *Helicobacter* infections, and this unresolved and sustained inflammation may lead to tissue damage and initiation of carcinogenesis and CCA (Sripa et al., 2012a, 2017). There have been several reviews that discuss the link between inflammatory responses and cancer, which may also be applicable to OV-induced CCA (Grivennikov et al., 2010; Balkwill and

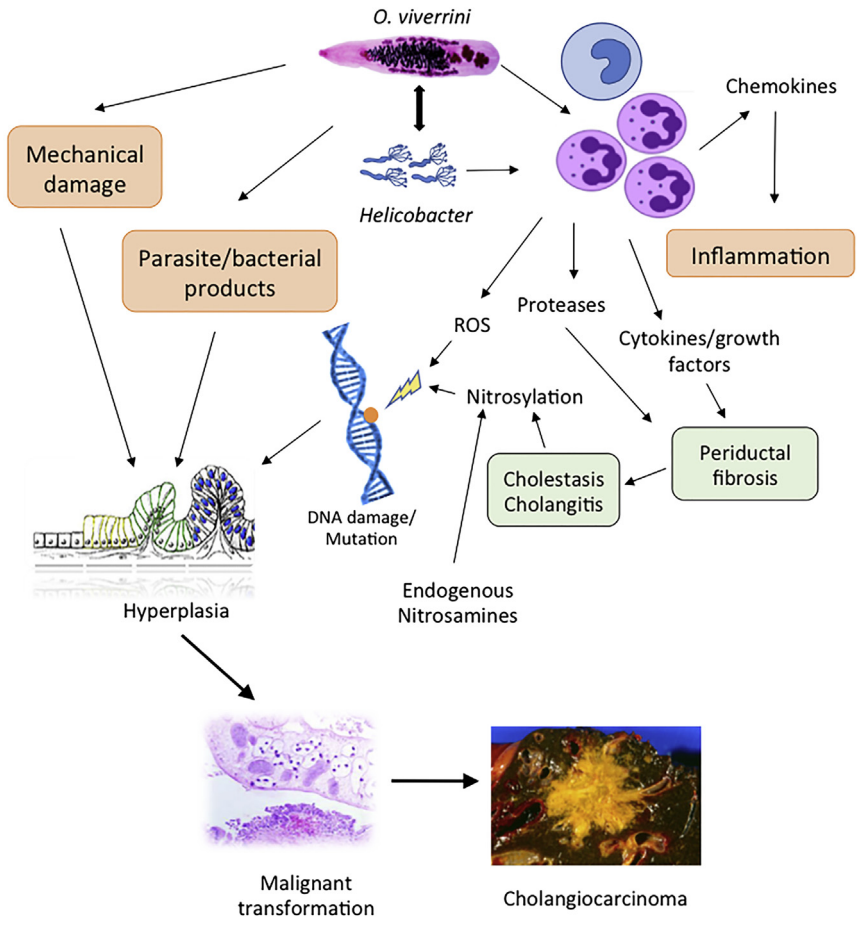

Figure 2 Proposed interplay between parasites, *Helicobacter* and inflammation in the events that lead to periductal fibrosis, DNA mutations, hyperplasia and malignant transformations that can lead to the development of cholangiocarcinoma. *Modified from Sripa, B., Deenonpoe, R., Brindley, P.J., 2017. Coinfections with liver fluke and* Helicobacter *species: a paradigm change in pathogenesis of opisthorchiasis and cholangiocarcinoma? Parasitol. Int. 66, 383–389.*

Mantovani, 2001; Lopez-Novoa and Nieto, 2009). Parallels may also be drawn with *H. pylori* infection, where almost 50% of the world's population are infected, yet only some develop the associated gastric cancer. Opisthorchiasis therefore represents another example of an inflammatory disease that elevates the possibility of cancer and may have some parallels with diseases such as rheumatoid arthritis and atherosclerosis (Chen et al., 2011; Pittet and Swirski, 2011).

2. IMMUNE ACTIVATION

2.1 Regulation of Immune Cell Activation During Helminth Infections

Inflammation in the context of helminth infection differs from other classical acute, inflammatory responses because helminths possess immunomodulatory properties. Helminths are capable of regulating the normal T helper 2 (Th2) responses against pathogens, mediated by immunoglobulin (Ig)E, eosinophils, mast cells and Th2 cytokines such as interleukin (IL)-4 and IL-5 (Maizels and Yazdanbakhsh, 2003; Maizels et al., 2014). Unlike microbial pathogen recognition, there are no clearly identified pathogen-associated molecular patterns on helminths that facilitate their recognition by pattern recognition receptors (PRRs) on immune cells (Perrigoue et al., 2008). Helminth-mediated immunomodulation is typically associated with increased antiinflammatory mediators such as IL-10 levels and Treg activity, a finding validated in mouse models (Wilson et al., 2005). Helminths modulate host immunity via induction of strong Th2 responses, while downregulation of type 1 inflammation modifying the intestinal environment to promote their survival (Maizels et al., 2004). High expression levels of OV defence proteins, including T265_13308, in the bile duct may be responsible for immunomodulation to enable continued parasite feeding (Sithithaworn et al., 2014). The resulting immunomodulation is thought to promote helminth survival within human hosts (Suttiprapa et al., 2008).

The infiltration of inflammatory cells to sites of infection and fibrotic tissue/tumours is a consistent observation in hamster models (Sripa and Kaewkes, 2000, 2002). The infiltration of immune cells to sites of OV infection suggests that OVES products may either directly or indirectly (via influencing host cells) generate chemoattractant molecules. Neutrophils are recruited to the infected bile ducts in the early stages of infection, followed by eosinophils and monocytes which then predominate (Bhamarapravati et al., 1978; Jittimanee et al., 2007; Wongratanacheewin et al., 2003). Damage-associated molecular pattern signalling from necrotic cells damaged by OV feeding may also stimulate the recruitment of inflammatory immune cells (Kono and Rock, 2008). Much research has focussed on identifying the extracellular vesicles and soluble secreted molecules from OV in culture (OVES products) (Chaiyadet et al., 2015b, 2017). An OVES product has been hypothesized to sequester signals from necrotic cells, allowing the

parasite to feed and possibly evade detection (Smout et al., 2009). This may be an example of immunomodulatory properties of OV (Wongratanacheewin et al., 1987), masking wound damage in an attempt to decrease acute immune responses. It is established that T cells are not required for the recruitment and infiltration of innate immune cells (Flavell and Flavell, 1986) but may mediate inflammatory responses within the biliary network during opisthorchiasis.

The infiltration and recruitment of inflammatory cells to the site of infection is likely to be influenced by soluble OVES molecules eliciting IL-6 and IL-8 release from cholangiocytes. These cytokines are released following Toll-like receptor (TLR)-4-mediated activation of nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) via myeloid differentiation primary response gene 88 (MyD88) (Ninlawan et al., 2010). It is likely that the production of chemoattractant (IL-8) chemokines and proinflammatory (IL-6) cytokines from these nonimmune cell cholangiocytes is driven by clathyrin-mediated endocytosis of vesicular OVES products (Chaiyadet et al., 2015a). Notably, these chemokines and cytokines released during OV infection (Jittimanee et al., 2007) are also likely to stimulate the influx of immune cells to the infection site, which then themselves generate chemoattractants. It would also be of interest to define how the complement cascade responds to OV, given that the cascade is crucial to alerting innate cells to infection and can generate its own immune cell chemoattractants, such as C5a or C3a.

2.2 Immune Cell Responses to OV/OVES Products

It is somewhat surprising that literature covering specific responses of innate inflammatory cells towards OV is rather limited, given that infiltration of these cells to the site of infection is a consistent observation in animal models of OV infection. Eosinophilia is associated with OV infection (Nishiura et al., 2003) and is common in other parasitic infections (Shin et al., 2009). In the case of OV, there is early infiltration of neutrophils and both eosinophils and macrophages are persistently abundant around the site of infections after several weeks and months (Bhamarapravati et al., 1978; Sripa and Kaewkes, 2000). Therefore, it would be important to determine the specific responses of eosinophils and macrophages to OV/OVES products. This may reveal if innate responses are regulated directly by OV/OVES products and whether such a mechanism could explain the inflammatory damage observed in opisthorchiasis.

We have made the recent and novel discovery that circulating blood neutrophils from OV-infected individuals have a greater capacity to produce

reactive oxygen metabolites ROS (reactive oxygen species) and undergo phagocytosis than healthy controls (unpublished observations). These circulating neutrophils also have elevated surface expression of the integrin, CD11b. Moreover, neutrophils from OV-infected individuals with advanced periductal fibrosis have an even greater capacity to generate ROS and phagocytose and are sensitized to the stimulatory effects of OVES. This previously unrecognized involvement of circulating neutrophils during OV infection, particularly in those with liver disease, indicates a systemic activation of the innate immune system and a role for these cells in the pathological events associated with persistent inflammation.

Recently, significant increases in a number of proinflammatory and antiinflammatory cytokines were measured in isolated peripheral blood mononuclear cells (PBMCs) of non-OV-infected and OV-infected, CCA-positive patients stimulated with OVES products in vitro (Hongsrichan et al., 2014; Surapaitoon et al., 2017). Although it was not clear which cells were responsible for the cytokine release, i.e., T or B lymphocytes, monocytes or neutrophils, this research demonstrated that cytokine release from both unstimulated and stimulated PBMCs of OV-induced CCA patients was significantly elevated above levels in uninfected individuals. Whilst observations that individuals may acquire successive, repeated infections suggest inefficient innate and humoral responses that fail to clear OV (Flavell et al., 1980), this PBMC response to OV suggests a persistently activated state, increased sensitivity and perhaps even memory for responses to OV/OVES products. This may provide evidence of PBMCs from CCA patients becoming more responsive or 'primed' to OVES products, developing a form of memory that allows for a more rapid and greater release of cytokines. This 'prepared' state of PBMCs to release cytokines may regulate increased inflammatory responses to repeated infection and/or further release of OVES products, and it would be of great interest to determine if this 'primed' state also occurs in other immune cells. Alternatively, altered responses of PBMCs in CCA patients may be in response to the cancer. In the case of persistent infection, the upregulation of these cytokines (and perhaps other yet unidentified cytokines) will likely recruit immune cells and promote inflammatory responses in response to OV infection. It is therefore important to fully characterize these immune responses, including cytokine profiling, in other immune cells that are activated in OV infection, such as neutrophils and monocytes.

In the RAW264 macrophage-like cell line, OV antigen activates NF-κB-induced upregulation of inducible nitric oxide synthase (iNOS)

and cyclooxygenase-2 (COX-2) through TLR-2 (Pinlaor et al., 2005). This was comparable to responses of human peripheral blood leukocytes (PBLs), which also upregulated COX-2 and manganese superoxide dismutase 2 (MnSOD-2) and downregulated catalase (CAT) (Yongvanit et al., 2012a,b). However, it should be noted that this report did not specify which immune cells in the PBL population responded to OV antigen but does suggest that immune cells that possess TLR-2 receptors may be activated by OV antigen. Understanding which TLRs can be activated by OV will enhance our understanding of OV and provide insights into the biochemical composition of the parasite/antigen/OVES products and how comparable it is to that of other helminths/pathogens. Interestingly, while TLR-4 expression was not upregulated in the RAW264 cell line, it was significantly upregulated in cholangiocytes which could be responsible for enhanced IL-6 and IL-8 expression (Ninlawan et al., 2010). In addition, the upregulation of COX-2 suggests increased production of prostaglandins that may assist immune cell infiltration by stimulating processes such as vasodilation.

Both infiltrating and tissue-resident macrophages respond to OV (Pinlaor et al., 2009). However, the upregulation of iNOS in the RAW264 macrophage cell line is somewhat surprising, given that the anti-inflammatory and proliferative M2 phenotype of macrophages has been observed after chronic OV infection (Bility and Sripa, 2014; Thanee et al., 2015); this may represent differences in functions between RAW264 -cells and human macrophages. This M2 phenotype is associated with assisting wound healing processes which may be linked to fibrosis in chronic infections and promoted by the predominant Th2 response in OV-infected hamsters (Jittimanee et al., 2007). The M2 phenotype is not normally associated with proinflammatory characteristics, nor the inflammatory damage observed in opisthorchiasis. These latter phenotypes are more typical characteristics of the proinflammatory M1 phenotype. It would be of interest to determine if OV/OVES products generated at different stages of infection promote different subsets of each macrophage phenotype. Certainly, more research is required on this topic, as the concept of macrophage polarization in disease is still not well understood and requires further understanding.

2.3 OV-Induced Oxidative/Nitrative Damage and Survival of Parasites

The production of ROS/reactive nitrogen species (RNS) during a 'respiratory burst' is a key characteristic of inflammatory responses of cells such as neutrophils and macrophages during infection (Wright et al., 2010). The

presence and accumulation of 8-nitroguanine (Pinlaor et al., 2003) and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) lesions observed in the bile ducts of hamsters with repeated OV infection may be associated with immune cell iNOS production of nitric oxide (NO) (MacMicking et al., 1997; Pinlaor et al., 2004). These DNA lesions may also be a useful predictor of CCA (Saichua et al., 2015) because DNA damage increases the risk of mutations during cell division that precedes tumour cell formation. NO may also react with hydrogen peroxide (H_2O_2) produced by SOD-2, which is upregulated in PBLs exposed to OV antigen (Yongvanit et al., 2012a,b), to form peroxynitrite ($ONOO^-$), another free radical, thereby further increasing the risk of DNA damage.

Free radicals might not just be directed towards OV. The liver fluke is also believed to be a reservoir host for the known carcinogen, *H. pylori* (Deenonpoe et al., 2015), which, if it accesses the biliary network, could exacerbate inflammatory responses (Dangtakot et al., 2017) and even promote carcinogenesis, as it does in gastric cancer (Ajani et al., 2017). Free radicals may also target invading bacteria in the altered microbiome observed during opisthorchiasis (Plieskatt et al., 2013; Itthitaetrakool et al., 2016). This may represent another layer of complexity to OV infection, whereby immune cell infiltration may be in response to bacteria that were once commensal but have become pathogenic. The alteration of the microbiome and increases in fibrotic tissue in chronic infection are believed to limit the contractility of bile ducts. This, in turn, may allow the accumulation of bile sludge (Mairiang et al., 2012) that may pose an additional threat to the DNA of local cells.

An increased expression of the antioxidant peroxiredoxin-6 in inflammatory cells during opisthorchiasis (Khoontawad et al., 2010) indicates the host's attempt to protect itself from free radicals generated during infection. However, upregulation of oxidative enzymes and downregulation of catalase in PBLs may be responsible for the overall accumulation of free radicals and DNA damage in OV infection. Whilst cells possess DNA repair mechanisms that can limit or reverse DNA damage, an increased oxidative environment may overwhelm repair mechanisms, especially if OV can alter or even initiate mutations in these repair mechanisms (Tangkawattana et al., 2008; Jusakul et al., 2015). It is evident that OV can survive inflammatory responses, although observations following treatment with antiinflammatory drugs suggest the parasite is healthier when inflammatory responses are absent (Juasook et al., 2012). OV's ability to survive in this oxidative environment may be due to the ability of thioredoxin (Trx), identified in OV

and OVES products (Suttiprapa et al., 2008, 2012) to metabolize free radicals. This antioxidant may protect OV from oxidative damage. This is an example of how the parasite has adapted to protect itself from oxidative/nitrative damage, whilst local cells become damaged and hence are at risk of initiation of tumourigenesis.

3. HOW DOES OV INFECTION RESULT IN DEVELOPMENT OF CCA?

3.1 CCA: An OV-Induced Wound That Never Heals

Oxidative/nitrative damage is one mechanism by which inflammatory responses may directly initiate cancer. However, innate inflammatory responses may indirectly support tumourigenesis by promoting proliferative responses or inhibiting repair mechanisms during infection. Persistent OV infection prevents damaged cholangiocytes from fully repairing, which is a proposed characteristic of tumours (Dvorak, 1986). Usually, inflammatory cells leave sites of tissue damage or die by apoptosis once infection is cleared and then wound healing begins. However, persistent OV infection may cause inflammatory cells to remain throughout the infection (either by delayed apoptosis or by continuous rounds of infiltration), which will continue to release inflammatory and proliferative signals. The antiapoptotic activity of Trx in the OVES products has been described in cholangiocytes exposed to H_2O_2 in an oxidative stress model (Matchimakul et al., 2015). In addition to releasing free radicals and phagocytosing debris and necrotic cells, macrophages recruit fibroblasts via platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF-β); isoforms of both these cytokines are increased in response to OV infection (Smout et al., 2009; Boonjaraspinyo et al., 2012). Moreover, it is also recognized that activated neutrophils can generate locally high levels of proinflammatory and antiinflammatory cytokines and chemokines (Fig. 3), together with a range of growth factors and angiogenic mediators (Mantovani et al., 2011). High levels of IL-1β signalling (Pinlaor et al., 2005) from immune cells and increased collagen production and matrix metalloproteinases (MMPs) from recruited immune cells and fibroblasts are likely to result in an excess of extracellular matrix (ECM) required for tissue remodelling (Reviewed in Page-McCaw et al., 2007; Prakobwong et al., 2009). A role for MMPs in fibrosis-associated CCA in the hamster model has been proposed (Prakobwong et al., 2010) and MMP/TIMP

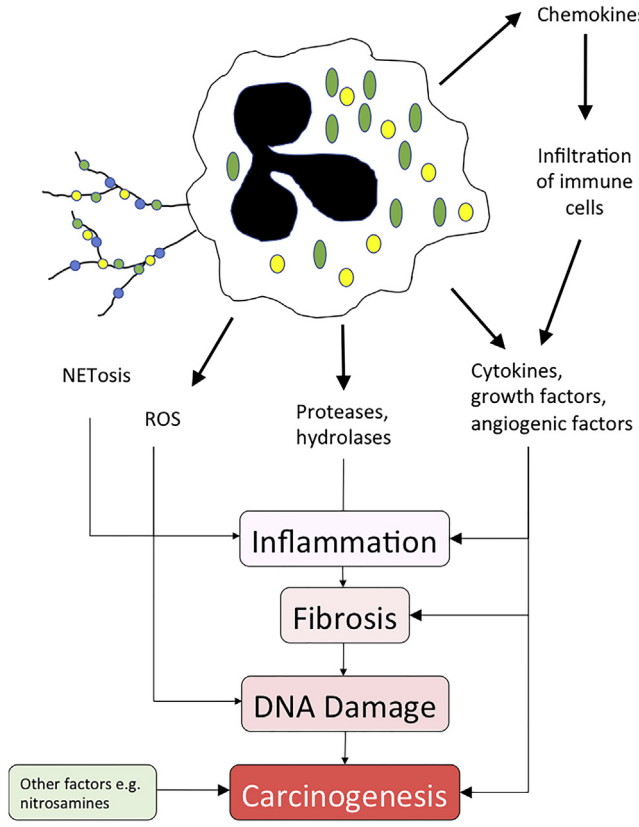

Figure 3 Neutrophil activation and generation/release of factors that can promote autoimmunity, inflammation and tissue damage and DNA damage. *ROS*, reactive oxygen species.

levels are elevated in OV-infected patients with CCA (Prakobwong et al., 2012). This continuous and excessive wound healing is likely to result in fibrotic tissue developing and promote an overproliferative phenotype in tissue cells, encouraging cells to replicate repetitively and uncontrollably. In addition, the proliferative granulin-like protein observed in OV (Smout et al., 2009) may also assist the wound healing response and tumour development.

Proliferative and proinflammatory cytokines upregulated in opisthorchiasis, such as IL-4, IL-6 and TGF-β, are required for wound healing processes, such as angiogenesis and cell proliferation. Specifically, IL-6, which is implicated in fibrosis and tumourigenesis (Fielding et al., 2014; Naugler and Karin, 2008), is associated with the development of APF and CCA in

opisthorchiasis (Sripa et al., 2009, 2012b; Frampton et al., 2012). However, this association is only observed in OV-infected individuals with APF and/or CCA, not in OV-infected individuals without APF and CCA. This suggests OV may not directly drive this IL-6 production, but APF/CCA may create the environment responsible for overproduction of IL-6. This implicates IL-6 as an indicator of CCA in OV-infected patients, which may be used to predict the onset of tumour formation, and this cytokine has previously been associated with cancers (Chiu et al., 1996; Wehbe et al., 2006). IL-6 may even increase the number of macrophages at sites of infection, encouraging further inflammatory and proliferative signalling. Inflammatory responses are likely to be responsible for the accumulation of fibrotic tissue, which can be identified via ultrasound. Because most OV-infected CCA patients present with fibrosis before the onset of the cancer, this further implicates inflammatory and fibrotic responses in the development of CCA (Sripa et al., 2012a).

Once a tumour has developed, it requires its own tumour microenvironment (TME) to survive. The TME often constitutes immune cells, fibroblasts, blood vessels, inflammatory cells, growth factors, and ECM: however, the TME is only partially defined in OV-induced CCA (Utispan et al., 2010; Chuaysri et al., 2009). The continued supply of growth factors, cytokines, nutrients and oxygen and enhanced vascularization during inflammatory/proliferative responses towards persistent OV infection are likely to help tumour progression. One component of the TME is tumour-associated macrophages (TAMs), which develop the M2 phenotype via processes that may be promoted by OV. This phenotype is considered protumourigenic and may support the TME for development of CCA and may even be implicated in tumour metastasis (Bility and Sripa, 2014). As the tumour establishes, it is likely to adopt a 'mutator' phenotype, characterized by increased genomic instability and erroneous DNA repair. Inflammatory cell release of free radicals during persistent OV infection may then allow mutations in DNA to occur more frequently. The chronic inflammation-associated upregulation of proliferative signalling pathways, phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) and Wnt/β-catenin signalling in OV-induced CCA tissues (Yothaisong et al., 2014) may support the mutator phenotype, allowing mutated cells to replicate quicker and more frequently, contributing to the overproliferative and uncontrolled replication often exhibited in tumours before they metastasize.

3.2 Genetic and Epigenetic Changes and Susceptibility to CCA

Whilst it is well established that host inflammation contributes to fibrosis and CCA as a result of OV infection, not every infected individual develops CCA (Sripa et al., 2012a). Of those infected in Thailand, only ~25% develop advanced periductal fibrosis, and ~1%–2% develop CCA (Mairiang et al., 2012). Whilst these are low percentages, because of the high number of people infected, this accounts for >26,000 CCA-associated deaths per year (Sripa and Pairojkul, 2008). The molecular basis for this heterogenous disease progression following infection is unknown.

Epigenetic alterations in host genomes are capable of causing susceptibility to various cancers and immune disorders and may play a role in the development of CCA. Whole-exome sequencing of the CCA genome (Jusakul et al., 2015) revealed novel gene mutations in CCA that are involved in chromatin function, including BAP1 (2.8%), ARID1A (17.6%), MLL3 (13%) and IDH1/2 (2.8%). Decreased expression and functionality of mismatch repair gene hMLH1 were also detected in 44.6% of OV-associated CCA (Jusakul et al., 2015). The detection of DNA methylation at novel CCA-mutated genes has been suggested as a biomarker for CCA (Jusakul et al., 2015). Aberrant DNA methylation is responsible for changes in cholangiocyte cell fate, favouring malignancy (Chiang et al., 2015). The identification of novel mutations in the CCA genome could allow for targeted DNA methylation analysis, using next generation sequencing, enabling rapid diagnosis of CCA (Masser et al., 2015). In addition, if identified gene mutations follow the two-hit or three-hit hypothesis towards CCA development, individuals at high risk of CCA development could be identified via mutated gene alleles (Knudson, 1971; Segditsas et al., 2009). The use of genome-wide association studies, as used to investigate disorders in immune function, could be applied to investigate dysregulated immunity in CCA (Knight, 2013).

However, it is also important to note that where carcinogenesis arises as a result of viruses, bacteria and other helminths, carcinogenesis usually occurs following a lifetime of infection allowing genetic alterations to accumulate over a time, ultimately leading to a malignant phenotype, as opposed to cancer development in those with a preexisting genetic predisposition (Smout et al., 2011).

4. *HELICOBACTER*: A CANCER-INDUCING BACTERIUM

4.1 *Helicobacter* and Gastric Disease

Helicobacter spp., especially *H. pylori*, has long been recognized as a risk factor for gastroduodenal diseases, including gastritis, peptic ulcer, gastric cancer and mucosa-associated lymphoid tissue lymphoma (Moss, 2017; Kao et al., 2016). Around 50% of the world's population are infected, and it has been classified by the WHO as a type I carcinogen. Most infected individuals have asymptomatic gastritis, but 10%–20% have an increased risk of developing peptic ulcers, and 1%–2% infected individuals have an increased risk of developing distal gastric cancer (Moss, 2017). Infection with *H. pylori* occurs in early life, and it may be beneficial in suppressing asthma, allergies and inflammatory bowel disease (Reibman et al., 2008; Chen and Blaser, 2008; Luther et al., 2010; Koch and Muller, 2015). Host gene polymorphisms may, at least in part, account for these varied pathological outcomes following infection. For example, IL-1β polymorphisms resulting in enhanced levels of expression of this molecule may predispose to gastric cancer as this molecule inhibits acid secretion that may lead to gastric atrophy, metaplasia and then gastric cancer, as well as inducing proliferation of gastric carcinoma cells (Hwang et al., 2002). IL-1β is therefore one of the key host factors for *H. pylori*–associated gastric cancer progression.

4.2 *Helicobacter* and Inflammation

H. pylori infection is associated with a large infiltration of neutrophils (together with monocytes and some other immune cells), which leads to gastritis, and there is a strong correlation between bacterial load and neutrophil numbers/inflammation scores (Xu et al., 2012). Neutrophil inflammation resolves following successful antibiotic treatment. In addition to neutrophil infiltration, there is evidence of neutrophil-mediated ROS production and myeloperoxidase release within the gastric epithelium (Davies et al., 1994; Fu et al., 2016), and these molecules may contribute to the tissue damage and events associated with the various gastric pathologies, including gastric cancer.

H. pylori colonization and pathogenicity is dependent on a number of key genes and proteins. These are (Kao et al., 2016) as follows: (1) urease, which is responsible for the conversion of urea to ammonia, may help in generating a localized, nonacidic microenvironment around the bacteria to promote their survival in the stomach. This enzyme may also affect the pH of phagosomes following phagocytosis by neutrophils and hence

interfere with bacterial killing processes; (2) a 40-kbp pathogenicity island in the more virulent strains (CagPAI) is a type IV secretory system that effectively delivers CagA protein into host cells to regulate a variety of intracellular signaling systems in the target cells; (3) vacuolating toxin A (VacA) induces toxicity in epithelial and immune cells; and (4) the bacteria also express a number of surface adhesion proteins that promote binding to gastric epithelial cells and they are highly motile.

One of the first *H. pylori* molecules to be characterized was HP-NAP (neutrophil-activating protein). It was first shown that a water-soluble molecule could be isolated from *H. pylori* that was capable of activating ROS production and chemotaxis in neutrophils (Mooney et al., 1991). This was later purified and characterized as HP-NAP, which is a 150-kDa molecule comprising 10 identical 15-kDa subunits (Evans et al., 1995). The NAP gene is present in all *Helicobacter* strains, but there is considerable heterogeneity in expression levels between strains. The 3D structure of HP-NAP resembles that of bacterioferritins (Tsuruta et al., 2012), and it is highly conserved. HP-NAP is normally present in the bacterial cytosol, and it is likely released after lysis. It is chemotactic for neutrophils, monocytes and other immune cells, and in addition to its ability to stimulate ROS and myeloperoxidase release from neutrophils (Wang et al., 2008), it can induce the secretion of a variety of cytokines and chemokines that can play a role in immune activation and pathology. For example, it stimulates the secretion of TNFα, IL-8, CCL (Chemokine C−C motif ligand) 3, CCL4 from neutrophils and IL-6 and other cytokines from monocytes (Alvarez-Arellano et al., 2007; Amedei et al., 2006). It can also trigger IL-12/23 release from neutrophils and monocytes thereby promoting a Th1 response and inhibiting a Th2 response (D'Elios and Andersen, 2007; de Bernard and D'Elios, 2010).

Other *H. pylori* molecules that can regulate cytokine/chemokine release include Hsp (Heat Shock Protein) 60 (GroEL-like HspB) and Hsp10 (GroES-like HspA) that induce IL-8, TNFα and Gro expression via TLR2-mediated activation of NF-κB (Lin et al., 2009; Zhao et al., 2007). In addition to stimulating the expression of these proinflammatory molecules, the antiinflammatory cytokine, IL-10 may also be activated (Pachathundikandi and Backert, 2018), indicating complex regulatory mechanisms to fine tune the inflammatory process following neutrophil infiltration and activation.

4.3 The Inflammasome

The inflammasome is a multicomponent protein complex that becomes assembled in innate myeloid cells in response to external signals such as

pathogens and it regulates the processing and expression of inflammatory cytokines such as IL-1β and IL-18. Inflammasome formation (reviewed in Broz and Dixit, 2016) is often triggered by ligation of PRRs such as TLRs, C-type lectin receptors (CLRs) or intracellular NOD-like receptors (NLRs). Their precise structure can vary, but generally they comprise an NOD-like receptor (e.g., NLRP1, NLRP3 or NLRP4), pro-caspase-1 and apoptosis-associated speck-like protein containing a CARD (cascade activation and recruitment domain). Once assembled, pro-caspase-1 becomes activated and can cleave pro-IL-1β and pro-IL-18 to active forms, which, when released, can regulate a number of cell and tissue functions. Inflammasomes containing NLRP3 have been described following exposure of neutrophils or other innate immune cells to *H. pylori* and its products (Semper et al., 2014; Perez-Figueroa et al., 2016; Pachathundikandi and Backert, 2018).

Inflammasome formation requires ROS production, potassium efflux and lysosomal destabilization (Semper et al., 2014), and both priming and second signals are required. The processes by which *H. pylori* activates the inflammasome in human innate cells are becoming clarified, but there is some debate in the literature as to the precise mechanisms involved. In part, these differences may be explained by the different experimental systems used (e.g., blocking TLR antibodies). A consensus view is emerging that bacterial components (e.g., LPS, glycopeptides, flagellin, UreB) activate TLR2 and TLR4, which then result in NF-κB activation. This then activates transcription of NLPR3 and pro-IL-1β and pro-IL-18, and the expressed NLPR3 assembles with ASC (apoptosis-associated speck-like protein containing a CARD) and pro-caspase-1 to form the inflammasome. Activated caspase-1 can then cleave the pro-cytokines that are then released to activate immune cells and tissue cells. Experiments using *H. pylori* strains and mutants with altered expression of key components indicate that the CagPAI and VacA are required (but perhaps not CagA) for activation of inflammasome assembly in immune cells (Pachathundikandi and Backert, 2018; Semper et al., 2014).

In addition to triggering cytokine expression, ROS production and degranulation, it was recently shown that human neutrophils adopt a new phenotype, resembling the N1 phenotype (Whitmore et al., 2017) following phagocytosis of *H. pylori*. Following phagocytosis neutrophils become $CD62L^{dim}$, $CD11b^{bright}$, $CD66b^{bright}$ and $CD63^{bright}$, in addition generating proinflammatory cytokines. They also have delayed apoptosis and develop a hypersegmented nucleus (Whitmore et al., 2017). Experiments with

different strains of *H. pylori* indicate that HP-NAP, VacA, CagG and CagT are not required for the neutrophils to acquire this unusual phenotype that is sometimes observed in clinical biopsies.

4.4 *Helicobacter* and Liver Fluke Infections

Helicobacter spp. (including *H. pylori*, *H. hepaticus* and *H. bilis*) are detected in the bile (Fukuda et al., 2002), gallbladder (Chen et al., 2003) and liver tissue (Huang et al., 2004) of patients with a number of hepatobiliary diseases. *H. pylori* is also detected in patients with liver carcinoma and CCA (Huang et al., 2004; Pellicano et al., 2004; Xuan et al., 2006; Abu Al-Soud et al., 2008).

A systematic study of 87 patients with liver fluke–associated CCA, 53 with cholelithiasis (benign disease group) and healthy control specimens (from autopsies) identified *H. pylori* in 66.7%, 41.5% and 25.0% of patients in CCA, cholelithiasis and control groups ($P < .05$), respectively (Boonyanugomol et al., 2012). *H. bilis* was detected in 14.9% and 9.4% of the patients with CCA and cholelithiasis, respectively ($P < .05$), but this species was absent from the control group. The *cagA* gene was detected in 36.2% and 9.1% of patients with CCA and cholelithiasis, respectively ($P < .05$, Boonyanugomol et al., 2012).

Further links between *H. pylori* and OV infection have come from animal studies. For example, coinfection of Syrian hamsters with OV and *H. pylori* resulted in the most severe hepatobiliary abnormalities, including periductal fibrosis, cholangitis and bile duct hyperplasia and hence a lower survival rate, compared with animals infected with either pathogen alone or control groups (Dangtakot et al., 2017). The highest levels of IL-1, IL-6 and TNFα in liver tissues were also observed in the dual-infected group. A microbiome/metagenomic study of hamsters infected with OV identified several bacterial species in the livers of infected hamsters, including *H. pylori* and other *Helicobacter* spp. (Itthitaetrakool et al., 2016). There are suggestions that OV may serve as a reservoir for *H. pylori* (Deenonpoe et al., 2015), which could explain the presence of this cancer-inducing bacterium following OV infection. A recent large-scale study of 500 individuals showed that liver fluke infection was associated with a higher frequency of CagA-positive *H. pylori*, present in 64.6% of stool samples compared with a presence of only 29.6% in stool samples from uninfected controls (Deenonpoe et al., 2017). The CagPAI is also necessary for bacterial internalization in primary hepatobiliary cells derived from CCA patients (Boonyanugomol et al., 2013).

4.5 OV and Activation of Innate Immunity: The *Helicobacter* Link

The recent discovery of a link between *H. pylori* coinfection with OV infection has a number of implications for understanding disease pathology and possible new avenues for treatment (Sripa et al., 2017). It is tempting to speculate that *Helicobacter* coinfection can stimulate the innate immune system, by similar mechanisms that occur in gastric disease, to activate the immune system in opisthorchiasis and contribute to the events that lead to periductal fibrosis and CCA. *H. pylori*—derived factors may be responsible for directly or indirectly activating neutrophils in OV-infected individuals, which may contribute to the release of tissue-damaging molecules and generation of proinflammatory cytokines and chemokines.

5. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

OV has clear carcinogenic potential. Whether this is a direct result of promoting overinflammatory conditions, overproliferative responses or decreased repair mechanisms, CCA onset is triggered by OV. The literature to date indicates that various innate immune responses that are predicted to occur or have been measured during OV-induced inflammation can lead to cell damage that may initiate or trigger carcinogenesis. Whether OV directly causes cancer, or whether the hypothesized 'proinflammatory phenotype' is directly responsible for the onset of cancer or produces an environment for cancer to develop from other carcinogens, is still to be determined. Also, it is necessary to determine the role played by *Helicobacter* spp. in the events that lead to the development of CCA. In particular, it is necessary to determine if this bacterium directly activates the innate immune system in similar ways that it does in gastric diseases. It would therefore be of interest to determine if anti-*Helicobacter* antibiotic treatment will be beneficial in preventing the development of CCA following OV infection. It is possible that OV has evolved mechanisms to generate the inflammatory environment and cancer so that it then has access to a greater source of nutrients. However, the parasite itself is largely resistant to the attempts of the innate immune system to destroy and eliminate it. More research is required before the proinflammatory phenotype can be fully characterized. However, if this phenotype can be demonstrated, it may allow a better understanding of how infectious agents can drive inflammation-related cancer and perhaps other inflammatory

diseases. This may lead to the development of new therapeutic approaches perhaps drawing on analogies in diseases such as rheumatoid arthritis, where targeting of the immune system with specific biologic drugs lead to dramatic improvement in disease progression.

REFERENCES

Abu Al-Soud, W., Stenram, U., Ljungh, A., Tranberg, K.G., Nilsson, H.O., Wadstrom, T., 2008. DNA of *Helicobacter* spp. and common gut bacteria in primary liver carcinoma. Dig. Liver Dis. 40, 126–131.

Ajani, J.A., Lee, J., Sano, T., Janjigian, Y.Y., Fan, D.M., Song, S.M., 2017. Gastric adenocarcinoma. Nat. Rev. Dis. Primers 3. https://doi.org/10.1038/nrdp.2017.36.

Alvarez-Arellano, L., Camorlinga-Ponce, M., Maldonado-Bernal, C., Torres, J., 2007. Activation of human neutrophils with *Helicobacter pylori* and the role of Toll-like receptors 2 and 4 in the response. FEMS Immunol. Med. Microbiol. 51, 473–479.

Amedei, A., Cappon, A., Codolo, G., Cabrelle, A., Polenghi, A., Benagiano, M., Tasca, E., Azzurri, A., D'Elios, M.M., Del Prete, G., de Bernard, M., 2006. The neutrophil-activating protein of *Helicobacter pylori* promotes Th1 immune responses. J. Clin. Invest. 116, 1092–1101.

Andrews, R.H., Sithithaworn, P., Petney, T.N., 2008. *Opisthorchis viverrini*: an underestimated parasite in world health. Trends Parasitol. 24, 497–501.

Balkwill, F., Mantovani, A., 2001. Inflammation and cancer: back to Virchow? Lancet 357, 539–545.

Bartsch, H., Montesano, R., 1984. Relevance of nitrosamines to human cancer. Carcinogenesis 5, 1381–1393.

Bhamarapravati, N., Thammavit, W., Vajrasthira, S., 1978. Liver changes in hamsters infected with a liver fluke of man, *Opisthorchis viverrini*. Am. J. Trop. Med. Hyg. 27, 787–794.

Bility, M.T., Sripa, B., 2014. Chronic *Opisthorchis viverrini* infection and associated hepatobiliary disease is associated with iron loaded M2-like macrophages. Kor. J. Parasitol. 52, 695–699.

Boonjaraspinyo, S., Wu, Z., Boonmars, T., Kaewkes, S., Loilome, W., Sithithaworn, P., Nagano, I., Takahashi, Y., Yongvanit, P., Bhudhisawasdi, V., 2012. Overexpression of PDGFA and its receptor during carcinogenesis of *Opisthorchis viverrini*-associated cholangiocarcinoma. Parasitol. Int. 61, 145–150.

Boonmars, T., Wu, Z., Boonjaruspinyo, S., Puapairoj, A., Kaewsamut, B., Nagano, I., Pinlaor, S., Yongvanit, P., Wonkchalee, O., Juasook, A., Sudsarn, P., Srisawangwong, T., 2011. Involvement of c-Ski oncoprotein in carcinogenesis of cholangiocarcinoma induced by *Opisthorchis viverrini* and N-nitrosodimethylamine. Pathol. Oncol. Res. 17, 219–227.

Boonyanugomol, W., Chomvarin, C., Hahnvajanawong, C., Sripa, B., Kaparakis-Liaskos, M., Ferrero, R.L., 2013. *Helicobacter pylori* cag pathogenicity island (cagPAI) involved in bacterial internalization and IL-8 induced responses via NOD1- and MyD88-dependent mechanisms in human biliary epithelial cells. PLoS One 8, e77358.

Boonyanugomol, W., Chomvarin, C., Sripa, B., Bhudhisawasdi, V., Khuntikeo, N., Hahnvajanawong, C., Chamsuwan, A., 2012. *Helicobacter pylori* in Thai patients with cholangiocarcinoma and its association with biliary inflammation and proliferation. HPB 14, 177–184.

Broz, P., Dixit, V.M., 2016. Inflammasomes: mechanism of assembly, regulation and signaling. Nat. Rev. Immunol. 16, 407–420.

Chaiyadet, S., Smout, M., Johnson, M., Whitchurch, C., Turnbull, L., Kaewkes, S., Sotillo, J., Loukas, A., Sripa, B., 2015a. Excretory/secretory products of the carcinogenic liver fluke are endocytosed by human cholangiocytes and drive cell proliferation and IL6 production. Int. J. Parasitol. 45, 773–781.

Chaiyadet, S., Smout, M., Laha, T., Sripa, B., Loukas, A., Sotillo, J., 2017. Proteomic characterization of the internalization of *Opisthorchis viverrini* excretory/secretory products in human cells. Parasitol. Int. 66, 494–502.

Chaiyadet, S., Sotillo, J., Smout, M., Cantacessi, C., Jones, M.K., Johnson, M.S., Turnbull, L., Whitchurch, C.B., Potriquet, J., Laohaviroj, M., Mulvenna, J., Brindley, P.J., Bethony, J.M., Laha, T., Sripa, B., Loukas, A., 2015b. Carcinogenic liver fluke secretes extracellular vesicles that promote cholangiocytes to adopt a tumorigenic phenotype. J. Infect. Dis. 212, 1636–1645.

Chen, W., Li, D., Cannan, R.J., Stubbs, R.S., 2003. Common presence of *Helicobacter* DNA in the gallbladder of patients with gallstone diseases and controls. Dig. Liver Dis. 35, 237–243.

Chen, Y.J., Chang, Y.T., Wang, C.B., Wu, C.Y., 2011. The risk of cancer in patients with rheumatoid arthritis: a nationwide cohort study in Taiwan. Arthritis Rheum. 63, 352–358.

Chen, Y., Blaser, M.J., 2008. *Helicobacter pylori* colonization is inversely associated with childhood asthma. J. Infect. Dis. 198, 553–560.

Chiang, N.J., Shan, Y.S., Hung, W.C., Chen, L.T., 2015. Epigenetic regulation in the carcinogenesis of cholangiocarcinoma. Int. J. Biochem. Cell Biol. 67, 110–114.

Chiu, J.J., Sgagias, M.K., Cowan, K.H., 1996. Interleukin 6 acts as a paracrine growth factor in human mammary carcinoma cell lines. Clin. Canc. Res. 2, 215–221.

Chuaysri, C., Thuwajit, P., Paupairoj, A., Chau-In, S., Suthiphongchai, T., Thuwajit, C., 2009. Alpha-smooth muscle actin-positive fibroblasts promote biliary cell proliferation and correlate with poor survival in cholangiocarcinoma. Oncol. Rep. 21, 957–969.

D'Elios, M.M., Andersen, L.P., 2007. *Helicobacter pylori* inflammation, immunity, and vaccines. Helicobacter 12, 15–19.

Dangtakot, R., Pinlaor, S., Itthitaetrakool, U., Chaidee, A., Chomvarin, C., Sangka, A., Wilailuckana, C., Pinlaor, P., 2017. Coinfection with *Helicobacter pylori* and *Opisthorchis viverrini* enhances the severity of hepatobiliary abnormalities in hamsters. Infect. Immun. 85, e00009–e00017.

Davies, G.R., Simmonds, N.J., Stevens, T.R., Sheaff, M.T., Banatvala, N., Laurenson, I.F., Blake, D.R., Rampton, D.S., 1994. *Helicobacter pylori* stimulates antral mucosal reactive oxygen metabolite production in vivo. Gut 35, 179–185.

de Bernard, M., D'Elios, M.M., 2010. The immune modulating activity of the *Helicobacter pylori* HP-NAP: friend or foe? Toxicon 56, 1186–1192.

Deenonpoe, R., Chomvarin, C., Pairojkul, C., Chamgramol, Y., Loukas, A., Brindley, P.J., Sripa, B., 2015. The carcinogenic liver fluke *Opisthorchis viverrini* is a reservoir for species of Helicobacter. Asian Pac. J. Cancer Prev. APJCP 16, 1751–1758.

Deenonpoe, R., Mairiang, E., Mairiang, P., Pairojkul, C., Chamgramol, Y., Rinaldi, G., Loukas, A., Brindley, P.J., Sripa, B., 2017. Elevated prevalence of *Helicobacter* species and virulence factors in opisthorchiasis and associated hepatobiliary disease. Sci. Rep. 7, 42744.

Dvorak, H.F., 1986. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. N. Engl. J. Med. 315, 1650–1659.

Evans, D.J., Evans, D.G., Takemura, T., Nakano, H., Lampert, H.C., Graham, D.Y., Granger, D.N., Kvietys, P.R., 1995. Characterization of a *Helicobacter-pylori* neutrophil-activating protein. Infect. Immun. 63, 2213–2220.

Fielding, C.A., Jones, G.W., McLoughlin, R.M., McLeod, L., Hammond, V.J., Uceda, J., Williams, A.S., Lambie, M., Foster, T.L., Liao, C.T., Rice, C.M., Greenhill, C.J., Colmont, C.S., Hams, E., Coles, B., Kift-Morgan, A., Newton, Z., Craig, K.J., Williams, J.D., Williams, G.T., Davies, S.J., Humphreys, I.R., O'Donnell, V.B., Taylor, P.R., Jenkins, B.J., Topley, N., Jones, S.A., 2014. Interleukin-6 signaling drives fibrosis in unresolved inflammation. Immunity 40, 40–50.

Flavell, D.J., Flavell, S.U., 1986. *Opisthorchis viverrini*: pathogenesis of infection in immunodeprived hamsters. Parasite Immunol. 8, 455–466.

Flavell, D.J., Pattanapanyasat, K., Flavell, S.U., 1980. *Opisthorchis viverrini*: partial success in adoptively transferring immunity with spleen cells and serum in the hamster. J. Helminthol. 54, 191–197.

Frampton, G., Invernizzi, P., Bernuzzi, F., Pae, H.Y., Quinn, M., Horvat, D., Galindo, C., Huang, L., McMillin, M., Cooper, B., Rimassa, L., DeMorrow, S., 2012. Interleukin-6-driven progranulin expression increases cholangiocarcinoma growth by an Akt-dependent mechanism. Gut 61, 268–277.

Fu, H., Ma, Y., Yang, M., Zhang, C., Huang, H., Xia, Y., Lu, L., Jin, W., Cui, D., 2016. Persisting and increasing neutrophil infiltration associates with gastric carcinogenesis and E-cadherin downregulation. Sci. Rep. 6, 29762.

Fukuda, K., Kuroki, T., Tajima, Y., Tsuneoka, N., Kitajima, T., Matsuzaki, S., Furui, J., Kanematsu, T., 2002. Comparative analysis of *Helicobacter* DNAs and biliary pathology in patients with and without hepatobiliary cancer. Carcinogenesis 23, 1927–1931.

Ghouri, Y.A., Mian, I., Blechacz, B., 2015. Cancer review: cholangiocarcinoma. J. Carcinog. 14, 1. https://doi.org/10.4103/1477-3163.151940.

Grivennikov, S.I., Greten, F.R., Karin, M., 2010. Immunity, inflammation, and cancer. Cell 140, 883–899.

Hongsrichan, N., Intuyod, K., Pinlaor, P., Khoontawad, J., Yongvanit, P., Wongkham, C., Roytrakule, S., Pinlaora, S., 2014. Cytokine/chemokine secretion and proteomic identification of upregulated annexin A1 from peripheral blood mononuclear cells cocultured with the liver fluke *Opisthorchis viverrini*. Infect. Immun. 82, 2135–2147.

Huang, Y., Fan, X.G., Wang, Z.M., Zhou, J.H., Tian, X.F., Li, N., 2004. Identification of helicobacter species in human liver samples from patients with primary hepatocellular carcinoma. J. Clin. Pathol. 57, 1273–1277.

Hwang, I.R., Kodama, T., Kikuchi, S., Sakai, K., Peterson, L.E., Graham, D.Y., Yamaoka, Y., 2002. Effect of interleukin 1 polymorphisms on gastric mucosal interleukin 1 beta production in *Helicobacter pylori* infection. Gastroenterology 123, 1793–1803.

IARC, 2011. IARC. Monographs on the Evaluation of Carcinogenic Risks to Humans, Volume 100. A Review of Carcinogen—Part B: Biological Agents. International Agency for Research on Cancer, Lyon.

Itthitaetrakool, U., Pinlaor, P., Pinlaor, S., Chomvarin, C., Dangtakot, R., Chaidee, A., Wilailuckana, C., Sangka, A., Lulitanond, A., Yongvanit, P., 2016. Chronic *Opisthorchis viverrini* infection changes the liver microbiome and promotes *Helicobacter* growth. PLoS One 11, e0165798.

Jittimanee, J., Sermswan, R.W., Puapairoj, A., Maleewong, W., Wongratanacheewin, S., 2007. Cytokine expression in hamsters experimentally infected with *Opisthorchis viverrini*. Parasite Immunol. 29, 159–167.

Juasook, A., Boonmars, T., Kaewkes, S., Loilome, W., Veteewuthacharn, K., Wu, Z., Yongvanit, P., 2012. Anti-inflammatory effect of prednisolone on the growth of human liver fluke in experimental opisthorchiasis. Parasitol. Res. 110, 2271–2279.

Jusakul, A., Kongpetch, S., Teh, B.T., 2015. Genetics of *Opisthorchis viverrini*-related cholangiocarcinoma. Curr. Opin. Gastroenterol. 31, 258–263.

Kao, C.Y., Sheu, B.S., Wu, J.J., 2016. *Helicobacter pylori* infection: an overview of bacterial virulence factors and pathogenesis. Biomed. J. 39, 14–23.

Khoontawad, J., Wongkham, C., Hiraku, Y., Yongvanit, P., Prakobwong, S., Boonmars, T., Pinlaor, P., Pinlaor, S., 2010. Proteomic identification of peroxiredoxin 6 for host defence against *Opisthorchis viverrini* infection. Parasite Immunol. 32, 314–323.

Knight, J.C., 2013. Genomic modulators of the immune response. Trends Genet. 29, 74–83.

Knudson Jr., A.G., 1971. Mutation and cancer: statistical study of retinoblastoma. Proc. Natl. Acad. Sci. U. S. A. 68, 820–823.

Koch, K.N., Muller, A., 2015. *Helicobacter pylori* activates the TLR2/NLRP3/caspase-1/IL-18 axis to induce regulatory T-cells, establish persistent infection and promote tolerance to allergens. Gut Microb. 6, 382–387.

Kono, H., Rock, K.L., 2008. How dying cells alert the immune system to danger. Nature Rev. Immunol. 8, 279–289.

Lim, J.H., 2011. Liver flukes: the malady neglected. Korean J. Radiol. 12, 269–279.

Lin, C.Y., Huang, Y.S., Li, C.H., Hsieh, Y.T., Tsai, N.M., He, P.J., Hsu, W.T., Yeh, Y.C., Chiang, F.H., Wu, M.S., Chang, C.C., Liao, K.W., 2009. Characterizing the polymeric status of *Helicobacter pylori* heat shock protein 60. Biochem. Biophys. Res. Commun. 388, 283–289.

Lopez-Novoa, J.M., Nieto, M.A., 2009. Inflammation and EMT: an alliance towards organ fibrosis and cancer progression. EMBO Mol. Med. 1, 303–314.

Luther, J., Dave, M., Higgins, P.D., Kao, J.Y., 2010. Association between *Helicobacter pylori* infection and inflammatory bowel disease: a meta-analysis and systematic review of the literature. Inflamm. Bowel Dis. 16, 1077–1084.

Luvira, V., Nilprapha, K., Bhudhisawasdi, V., Pugkhem, A., Chamadol, N., Kamsa-ard, S., 2016. Cholangiocarcinoma patient outcome in Northeastern Thailand: single-center prospective study. Asian Pac. J. Cancer Prev. APJCP 17, 401–406.

MacMicking, J., Xie, Q.W., Nathan, C., 1997. Nitric oxide and macrophage function. Annu. Rev. Immunol. 15, 323–350.

Mairiang, E., Laha, T., Bethony, J.M., Thinkhamrop, B., Kaewkes, S., Sithithaworn, P., Tesana, S., Loukas, A., Brindley, P.J., Sripa, B., 2012. Ultrasonography assessment of hepatobiliary abnormalities in 3359 subjects with *Opisthorchis viverrini* infection in endemic areas of Thailand. Parasitol. Int. 61, 208–211.

Maizels, R.M., Yazdanbakhsh, M., 2003. Immune regulation by helminth parasites: cellular and molecular mechanisms. Nat. Rev. Immunol. 3, 733–744.

Maizels, R.M., Balic, A., Gomez-Escobar, N., Nair, M., Taylor, M.D., Allen, J.E., 2004. Helminth parasites–masters of regulation. Immunol. Rev. 201, 89–116.

Maizels, R.M., McSorley, H.J., Smyth, D.J., 2014. Helminths in the hygiene hypothesis: sooner or later? Clin. Exp. Immunol. 177, 38–46.

Mantovani, A., Cassatella, M.A., Costantini, C., Jaillon, S., 2011. Neutrophils in the activation and regulation of innate and adaptive immunity. Nat. Rev. Immunol. 11, 519–531.

Masser, D.R., Stanford, D.R., Freeman, W.M., 2015. Targeted DNA methylation analysis by next-generation sequencing. JoVE 96. https://doi.org/10.3791/52488.

Matchimakul, P., Rinaldi, G., Suttiprapa, S., Mann, V.H., Popratiloff, A., Laha, T., Pimenta, R.N., Cochran, C.J., Kaewkes, S., Sripa, B., Brindley, P.J., 2015. Apoptosis of cholangiocytes modulated by thioredoxin of carcinogenic liver fluke. Int. J. Biochem. Cell Biol. 65, 72–80.

McSorley, H.J., Hewitson, J.P., Maizels, R.M., 2013. Immunomodulation by helminth parasites: defining mechanisms and mediators. Int. J. Parasitol. 43, 301–310.

Mooney, C., Keenan, J., Munster, D., Wilson, I., Allardyce, R., Bagshaw, P., Chapman, B., Chadwick, V., 1991. Neutrophil activation by *Helicobacter pylori*. Gut 32, 853–857.

Moss, S.F., 2017. The clinical evidence linking *Helicobacter pylori* to gastric cancer. Cell Mol. Gastroenterol. Hepatol. 3, 183–191.

Murata, M., Thanan, R., Ma, N., Kawanishi, S., 2012. Role of nitrative and oxidative DNA damage in inflammation-related carcinogenesis. J. Biomed. Biotechnol. 2012, 623019.

Naugler, W.E., Karin, M., 2008. The wolf in sheep's clothing: the role of interleukin-6 in immunity, inflammation and cancer. Trends Mol. Med. 14, 109–119.

Ninlawan, K., O'Hara, S.P., Splinter, P.L., Yongvanit, P., Kaewkes, S., Surapaitoon, A., LaRusso, N.F., Sripa, B., 2010. *Opisthorchis viverrini* excretory/secretory products induce toll-like receptor 4 upregulation and production of interleukin 6 and 8 in cholangiocyte. Parasitol. Int. 59, 616–621.

Nishiura, H., Tsunoda, T., Akao, N., 2003. A case of severe eosinophilia in the acute phase of *Opisthorchis viverrini* infection. Kansenshogaku Zasshi 77, 677–681.

Pachathundikandi, S.K., Backert, S., 2018. *Helicobacter pylori* controls NLRP3 expression by regulating hsa-miR-223-3p and IL-10 in cultured and primary human immune cells. Innate Immun. 24, 11–23.

Page-McCaw, A., Ewald, A.J., Werb, Z., 2007. Matrix metalloproteinases and the regulation of tissue remodeling. Nat. Rev. Mol. Cell Biol. 8, 221–233.

Pellicano, R., Mazzaferro, V., Grigioni, W.F., Cutufia, M.A., Fagoonee, S., Silengo, L., Rizzetto, M., Ponzetto, A., 2004. *Helicobacter* species sequences in liver samples from patients with and without hepatocellular carcinoma. World J. Gastroenterol. 10, 598–601.

Perez-Figueroa, E., Torres, J., Sanchez-Zauco, N., Contreras-Ramos, A., Alvarez-Arellano, L., Maldonado-Bernal, C., 2016. Activation of NLRP3 inflammasome in human neutrophils by *Helicobacter pylori* infection. Innate Immun. 22, 103–112.

Perrigoue, J.G., Marshall, F.A., Artis, D., 2008. On the hunt for helminths: innate immune cells in the recognition and response to helminth parasites. Cell Microbiol. 10, 1757–1764.

Pinlaor, P., Kaewpitoon, N., Laha, T., Sripa, B., Kaewkes, S., Morales, M.E., Mann, V.H., Parriott, S.K., Suttiprapa, S., Robinson, M.W., To, J., Dalton, J.P., Loukas, A., Brindley, P.J., 2009. Cathepsin F cysteine protease of the human liver fluke, *Opisthorchis viverrini*. PLoS Neglected Trop. Dis. 3, e398.

Pinlaor, S., Ma, N., Hiraku, Y., Yongvanit, P., Semba, R., Oikawa, S., Murata, M., Sripa, B., Sithithaworn, P., Kawanishi, S., 2004. Repeated infection with *Opisthorchis viverrini* induces accumulation of 8-nitroguanine and 8-oxo-7,8-dihydro-2'-deoxyguanine in the bile duct of hamsters via inducible nitric oxide synthase. Carcinogenesis 25, 1535–1542.

Pinlaor, S., Tada-Oikawa, S., Hiraku, Y., Pinlaor, P., Ma, N., Sithithaworn, P., Kawanishi, S., 2005. *Opisthorchis viverrini* antigen induces the expression of Toll-like receptor 2 in macrophage RAW cell line. Int. J. Parasitol. 35, 591–596.

Pinlaor, S., Yongvanit, P., Hiraku, Y., Ma, N., Semba, R., Oikawa, S., Murata, M., Sripa, B., Sithithaworn, P., Kawanishi, S., 2003. 8-nitroguanine formation in the liver of hamsters infected with *Opisthorchis viverrini*. Biochem. Biophys. Res. Commun. 309, 567–571.

Pittet, M.J., Swirski, F.K., 2011. Monocytes link atherosclerosis and cancer. Eur. J. Immunol. 41, 2519–2522.

Plieskatt, J.L., Deenonpoe, R., Mulvenna, J.P., Krause, L., Sripa, B., Bethony, J.M., Brindley, P.J., 2013. Infection with the carcinogenic liver fluke *Opisthorchis viverrini* modifies intestinal and biliary microbiome. FASEB J. 27, 4572–4584.

Prakobwong, S., Pinlaor, S., Yongvanit, P., Sithithaworn, P., Pairojkul, C., Hiraku, Y., 2009. Time profiles of the expression of metalloproteinases, tissue inhibitors of metalloproteases, cytokines and collagens in hamsters infected with *Opisthorchis viverrini* with special reference to peribiliary fibrosis and liver injury. Int. J. Parasitol. 39, 825–835.

Prakobwong, S., Yongvanit, P., Hiraku, Y., Pairojkul, C., Sithithaworn, P., Pinlaor, P., Pinlaor, S., 2010. Involvement of MMP-9 in peribiliary fibrosis and cholangiocarcinogenesis via Rac1-dependent DNA damage in a hamster model. Int. J. Canc. 127, 2576–2587.

Prakobwong, S., Charoensuk, L., Hiraku, Y., Pinlaor, P., Pairojkul, C., Mairiang, E., Sithithaworn, P., Yongvanit, P., Khuntikeo, N., Pinlaor, S., 2012. Plasma hydroxyproline, MMP-7 and collagen I as novel predictive risk markers of hepatobiliary disease-associated cholangiocarcinoma. Int. J. Canc. 131, E416–E424.

Reibman, J., Marmor, M., Filner, J., Fernandez-Beros, M.E., Rogers, L., Perez-Perez, G.I., Blaser, M.J., 2008. Asthma is inversely associated with *Helicobacter pylori* status in an urban population. PLoS One 3, e4060.

Saichua, P., Yakovleva, A., Kamamia, C., Jariwala, A.R., Sithithaworn, J., Sripa, B., Brindley, P.J., Laha, T., Mairiang, E., Pairojkul, C., Khuntikeo, N., Mulvenna, J., Sithithaworn, P., Bethony, J.M., 2015. Levels of 8-OxodG predict hepatobiliary pathology in *Opisthorchis viverrini* endemic settings in Thailand. PLoS Neglected Trop. Dis. 9, e0003949.

Segditsas, S., Rowan, A.J., Howarth, K., Jones, A., Leedham, S., Wright, N.A., Gorman, P., Chambers, W., Domingo, E., Roylance, R.R., Sawyer, E.J., Sieber, O.M., Tomlinson, I.P., 2009. APC and the three-hit hypothesis. Oncogene 28, 146–155.

Semper, R.P., Mejias-Luque, R., Gross, C., Anderl, F., Muller, A., Vieth, M., Busch, D.H., Prazeres da Costa, C., Ruland, J., Gross, O., Gerhard, M., 2014. *Helicobacter pylori*-induced IL-1beta secretion in innate immune cells is regulated by the NLRP3 inflammasome and requires the cag pathogenicity island. J. Immunol. 193, 3566–3576.

Shin, M.H., Lee, Y.A., Min, D.Y., 2009. Eosinophil-mediated tissue inflammatory responses in helminth infection. Kor. J. Parasitol. 47 (Suppl.), S125–S131.

Sithithaworn, P., Andrews, R.H., Nguyen, V.D., Wongsaroj, T., Sinuon, M., Odermatt, P., Nawa, Y., Liang, S., Brindley, P.J., Sripa, B., 2012a. The current status of opisthorchiasis and clonorchiasis in the Mekong Basin. Parasitol. Int. 61, 10–16.

Sithithaworn, P., Andrews, R.H., Petney, T.N., Saijuntha, W., Laoprom, N., 2012b. The systematics and population genetics of *Opisthorchis viverrini sensu lato*: implications in parasite epidemiology and bile duct cancer. Parasitol. Int. 61, 32–37.

Sithithaworn, P., Yongvanit, P., Duenngai, K., Kiatsopit, N., Pairojkul, C., 2014. Roles of liver fluke infection as risk factor for cholangiocarcinoma. J Hepatobiliary Pancreat. Sci. 21, 301–308.

Smout, M.J., Laha, T., Mulvenna, J., Sripa, B., Suttiprapa, S., Jones, A., Brindley, P.J., Loukas, A., 2009. A granulin-like growth factor secreted by the carcinogenic liver fluke, *Opisthorchis viverrini*, promotes proliferation of host cells. PLoS Pathog. 5, e1000611.

Smout, M.J., Sripa, B., Laha, T., Mulvenna, J., Gasser, R.B., Young, N.D., Bethony, J.M., Brindley, P.J., Loukas, A., 2011. Infection with the carcinogenic human liver fluke, *Opisthorchis viverrini*. Mol. Biosyst. 7, 1367–1375.

Sripa, B., Kaewkes, S., 2000. Localisation of parasite antigens and inflammatory responses in experimental opisthorchiasis. Int. J. Parasitol. 30, 735–740.

Sripa, B., Kaewkes, S., 2002. Gall bladder and extrahepatic bile duct changes in *Opisthorchis viverrini*-infected hamsters. Acta Trop. 83, 29–36.

Sripa, B., Pairojkul, C., 2008. Cholangiocarcinoma: lessons from Thailand. Curr. Opin. Gastroenterol. 24, 349–356.

Sripa, B., Brindley, P.J., Mulvenna, J., Laha, T., Smout, M.J., Mairiang, E., Bethony, J.M., Loukas, A., 2012a. The tumorigenic liver fluke *Opisthorchis viverrini*—multiple pathways to cancer. Trends Parasitol. 28, 395–407.

Sripa, B., Deenonpoe, R., Brindley, P.J., 2017. Co-infections with liver fluke and Helicobacter species: a paradigm change in pathogenesis of opisthorchiasis and cholangiocarcinoma? Parasitol. Int. 66, 383–389.

Sripa, B., Kaewkes, S., Sithithaworn, P., Mairiang, E., Laha, T., Smout, M., Pairojkul, C., Bhudhisawasdi, V., Tesana, S., Thinkamrop, B., Bethony, J.M., Loukas, A., Brindley, P.J., 2007. Liver fluke induces cholangiocarcinoma. PLoS Med. 4, e201.

Sripa, B., Mairiang, E., Thinkhamrop, B., Laha, T., Kaewkes, S., Sithithaworn, P., Tessana, S., Loukas, A., Brindley, P.J., Bethony, J.M., 2009. Advanced periductal fibrosis from infection with the carcinogenic human liver fluke *Opisthorchis viverrini* correlates with elevated levels of interleukin-6. Hepatology 50, 1273–1281.

Sripa, B., Tangkawattana, S., Laha, T., Kaewkes, S., Mallory, F.F., Smith, J.F., Wilcox, B.A., 2015. Toward integrated opisthorchiasis control in northeast Thailand: the Lawa project. Acta Trop. 141, 361–367.

Sripa, B., Thinkhamrop, B., Mairiang, E., Laha, T., Kaewkes, S., Sithithaworn, P., Periago, M.V., Bhudhisawasdi, V., Yonglitthipagon, P., Mulvenna, J., Brindley, P.J., Loukas, A., Bethony, J.M., 2012b. Elevated plasma IL-6 associates with increased risk of advanced fibrosis and cholangiocarcinoma in individuals infected by *Opisthorchis viverrini*. PLoS Neglected Trop. Dis. 6, e1654.

Surapaitoon, A., Suttiprapa, S., Khuntikeo, N., Pairojkul, C., Sripa, B., 2017. Cytokine profiles in *Opisthorchis viverrini* stimulated peripheral blood mononuclear cells from cholangiocarcinoma patients. Parasitol. Int. 66, 889–892.

Suttiprapa, S., Loukas, A., Laha, T., Wongkham, S., Kaewkes, S., Gaze, S., Brindley, P.J., Sripa, B., 2008. Characterization of the antioxidant enzyme, thioredoxin peroxidase, from the carcinogenic human liver fluke, *Opisthorchis viverrini*. Mol. Biochem. Parasitol. 160, 116–122.

Suttiprapa, S., Matchimakul, P., Loukas, A., Laha, T., Wongkham, S., Kaewkes, S., Brindley, P.J., Sripa, B., 2012. Molecular expression and enzymatic characterization of thioredoxin from the carcinogenic human liver fluke *Opisthorchis viverrini*. Parasitol. Int. 61, 101–106.

Tangkawattana, S., Kaewkes, S., Pairojkul, C., Tangkawattana, P., Sripa, B., 2008. Mutations of KRAS and TP53 in a minor proportion of *Opisthorchis viverrini*-associated cholangiocarcinomas in a hamster model. Asian Pac. J. Cancer Prev. APJCP 9, 101–106.

Thamavit, W., Bhamarapravati, N., Sahaphong, S., Vajrasthira, S., Angsubhakorn, S., 1978. Effects of dimethylnitrosamine on induction of cholangiocarcinoma in *Opisthorchis viverrini*-infected Syrian golden hamsters. Cancer Res. 38, 4634–4639.

Thanee, M., Loilome, W., Techasen, A., Namwat, N., Boonmars, T., Pairojkul, C., Yongvanit, P., 2015. Quantitative changes in tumor-associated M2 macrophages characterize cholangiocarcinoma and their association with metastasis. Asian Pac. J. Cancer Prev. APJCP 16, 3043–3050.

Tsuruta, O., Yokoyama, H., Fujii, S., 2012. A new crystal lattice structure of *Helicobacter pylori* neutrophil-activating protein (HP-NAP). Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun. 68, 134–140.

Utispan, K., Thuwajit, P., Abiko, Y., Charngkaew, K., Paupairoj, A., Chau-in, S., Thuwajit, C., 2010. Gene expression profiling of cholangiocarcinoma-derived fibroblast reveals alterations related to tumor progression and indicates periostin as a poor prognostic marker. Mol. Canc. 9, 13. https://doi.org/10.1186/1476-4598-9-13.

Wang, C.A., Liu, Y.C., Du, S.Y., Lin, C.W., Fu, H.W., 2008. *Helicobacter* pylori neutrophil-activating protein promotes myeloperoxidase release from human neutrophils. Biochem. Biophys. Res. Commun. 377, 52–56.

Wehbe, H., Henson, R., Meng, F., Mize-Berge, J., Patel, T., 2006. Interleukin-6 contributes to growth in cholangiocarcinoma cells by aberrant promoter methylation and gene expression. Cancer Res. 66, 10517–10524.

Whitmore, L.C., Weems, M.N., Allen, L.H., 2017. Cutting edge: *Helicobacter pylori* induces nuclear hypersegmentation and subtype differentiation of human neutrophils in vitro. J. Immunol. 198, 1793–1797.

Wilson, M.S., Taylor, M.D., Balic, A., Finney, C.A., Lamb, J.R., Maizels, R.M., 2005. Suppression of allergic airway inflammation by helminth-induced regulatory T cells. J. Exp. Med. 202, 1199–1212.

Wongratanacheewin, S., Rattanasiriwilai, W., Priwan, R., Sirisinha, S., 1987. Immunodepression in hamsters experimentally infected with *Opisthorchis viverrini*. J. Helminthol. 61, 151–156.
Wongratanacheewin, S., Sermswan, R.W., Sirisinha, S., 2003. Immunology and molecular biology of *Opisthorchis viverrini* infection. Acta Trop. 88, 195–207.
Wright, H.L., Moots, R.J., Bucknall, R.C., Edwards, S.W., 2010. Neutrophil function in inflammation and inflammatory diseases. Rheumatology 49, 1618–1631.
Xu, X.Q., Wang, Z.H., Liao, J.X., Chen, X.Y., Liu, W.Z., Xiao, S.D., Lu, H., 2012. Predictive value of neutrophil infiltration as a marker of *Helicobacter pylori* infection. World J. Gastroenterol. 18, 5101–5105.
Xuan, S.Y., Li, N., Qiang, X., Zhou, R.R., Shi, Y.X., Jiang, W.J., 2006. *Helicobacter* infection in hepatocellular carcinoma tissue. World J. Gastroenterol. 12, 2335–2340.
Yeoh, K.W., Promthet, S., Sithithaworn, P., Kamsa-Ard, S., Parkin, D.M., 2015. Re-examination of *Opisthorchis viverrini* infection in Northeast Thailand. Asian Pac. J. Cancer Prev. APJCP 16, 3413–3418.
Yongvanit, P., Pinlaor, S., Bartsch, H., 2012a. Oxidative and nitrative DNA damage: key events in opisthorchiasis-induced carcinogenesis. Parasitol. Int. 61, 130–135.
Yongvanit, P., Thanan, R., Pinlaor, S., Sithithaworn, P., Loilome, W., Namwat, N., Techasen, A., Dechakhamphu, S., 2012b. Increased expression of TLR-2, COX-2, and SOD-2 genes in the peripheral blood leukocytes of opisthorchiasis patients induced by *Opisthorchis viverrini* antigen. Parasitol. Res. 110, 1969–1977.
Yothaisong, S., Thanee, M., Namwat, N., Yongvanit, P., Boonmars, T., Puapairoj, A., Loilome, W., 2014. *Opisthorchis viverrini* infection activates the PI3K/AKT/PTEN and Wnt/beta-catenin signaling pathways in a cholangiocarcinogenesis model. Asian Pac. J. Cancer Prev. APJCP 15, 10463–10468.
Zhao, Y., Yokota, K., Ayada, K., Yamamoto, Y., Okada, T., Shen, L., Oguma, K., 2007. *Helicobacter pylori* heat-shock protein 60 induces interleukin-8 via a Toll-like receptor (TLR)2 and mitogen-activated protein (MAP) kinase pathway in human monocytes. J. Med. Microbiol. 56, 154–164.

FURTHER READING

Glaser, S.S., Gaudio, E., Miller, T., Alvaro, D., Alpini, G., 2009. Cholangiocyte proliferation and liver fibrosis. Expet Rev. Mol. Med. 11, e7.
Songserm, N., Prasongwattana, J., Sithithaworn, P., Sripa, B., Pipitkool, V., 2009. Cholangiocarcinoma in experimental hamsters with long-standing *Opisthorchis viverrini* infection. Asian Pac. J. Cancer Prev. APJCP 10, 299–302.