Implications of Secretory Leukocyte Protease Inhibitor Expression for Patient Classification in Inflammatory Bowel Disease and Colorectal Cancer

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The research described in this thesis was conducted at the Laboratory of Pediatrics, division Gastroenterology and Nutrition, Erasmus Medical Center in Rotterdam, the Netherlands.
The research described in this thesis was financially supported by the Dutch Digestive Foundation (grant registration number: Focus Project 15-17), the PIBD Network for Safety, Efficacy, Treatment and Quality improvement of care project funded by the European Commission Horizon 2020 (funding source number 668 023), and the collaborative TIMID project (LSHM18057-SGF).

Implications of Secretory Leukocyte Protease Inhibitor Expression for Patient Classification in Inflammatory Bowel Disease and Colorectal Cancer
ISBN: 978-94-6458-978-8

Provided by thesis specialist Ridderprint, ridderprint.nl
Printing: Ridderprint
Layout and design: Jeroen Reith, persoonlijkproefschrift.nl
Cover painting by Marian Nugteren; title painting: vulkaan (Eyjafjallajökull, Iceland).

Printing of this thesis was financially supported by Hycult Biotech and ChipSoft.

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Betekenis van Secretory Leukocyte Protease Inhibitor expressie voor patiënt classificatie in chronische darmontsteking en dikkedarmkanker

Thesis

to obtain the degree of Doctor from the
Erasmus University Rotterdam

by command of the
rector magnificus

Prof. dr. A.L. Bredenoord

and in accordance with the decision of the Doctorate Board.

The public defence shall be held on

Tuesday the 2nd of May 2023 at 10:30 hrs

by

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CHAPTER 1

General introduction and outline of this thesis

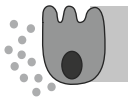
The intestinal epithelium is key in orchestrating a tailored intestinal immune response to the commensal microbiota

The intestine is continuously exposed to large amounts of microbial and dietary antigens. The number of bacteria ranges from 10^5 per ml in the proximal part of the small intestine to 10^{12} per ml in the colon (the large intestine), with the highest load in the distal part of the colon (1). Commensal bacteria are harmless under normal circumstances and are essential for the development and function of the intestine, as they are critical for digestion, produce essential metabolites, shape the mucosal immune system, and outcompete pathogenic micro-organisms (2-4). It is crucial that the intestinal immune response prevents tissue damaging pro-inflammatory immune responses to beneficial luminal commensals while mounting inflammatory host defense when commensals or pathogens breach the intestinal barrier and invade the tissue. Thus, a tightly regulated immune response tailored to the type and location of the micro-organism is required for intestinal homeostasis.

Key to this tailored immune response is the single layer of columnar epithelial cells that lines the intestinal mucosa and provides a barrier to intestinal commensals (5). In the small intestine, where breakdown and absorption of ingested nutrients occurs, the surface area is increased by mucosal fingerlike projections called villi. Below the villi lie tubular intestinal glands called crypts of Lieberkühn. The colon, where absorption of water occurs, only has crypts and no villi. At the basis of the crypts the pluripotent stem cells are located, which give rise to the all types of differentiated intestinal epithelial cells and are responsible for the continuous renewal of the epithelium (6). The main epithelial cells lining the villi and the crypts are absorptive cells. Interspersed among the absorptive cells are mucus-producing goblet cells, which are especially numerous in the crypts of the distal colon. In the small intestine, Paneth cells are located at the basis of the crypts and produce antimicrobial peptides and protect the stem cells (7,8). Finally, enteroendocrine cells secrete hormones important for digestion and M cells transport antigens from the lumen to organized mucosal lymphoid tissues.

Intestinal epithelial cells limit the contact between commensal bacteria and immune cells in several ways. First, the layer of mucus formed by goblet cells coats the mucosa and acts as a physical barrier. In addition, mucus contains glycoproteins which are toxic to many bacteria (1). In the colon, the mucus layer is composed of an outer layer where bacteria can be found and an inner layer which is normally free of bacteria (9). Absence of the major mucin forming the human and murine mucus layer, mucin2, allows close contact between normally harmless resident bacteria and the colonic epithelium and leads to spontaneous colitis (9,10). In addition, secretion of antimicrobial peptides by intestinal epithelial cells prevents micro-organisms from reaching the epithelial barrier

(11). In particular, in the small intestinal crypts Paneth cells secrete various antimicrobial peptides, thereby preventing the presence of micro-organisms in the deeper crypt regions. Moreover, the intestinal epithelium forms a physical barrier between micro-organisms and the lamina propria, the area directly underneath the epithelium where most intestinal monocytes, macrophages and lymphocytes reside. Intestinal epithelial cells are connected by tight junctions, which are impermeable to most bacteria, and are separated from the lamina propria by the basement membrane, a dense extracellular matrix which cannot be breached by most bacteria. Finally, intestinal epithelial cells transport large amounts of secretory IgA produced by plasma B cells from the lamina propria to the lumen, where IgA binds to bacteria inhibiting their adherence to the epithelium (12). Together, these mechanisms ensure that commensal micro-organisms are contained within the lumen and that their contact with the epithelium is limited during steady state.



Apart from forming a physical and biochemical barrier, intestinal epithelial cells also regulate the initiation of immune responses upon microbial contact. Innate immune cells, including intestinal epithelial cells, recognize microbe-associated molecular patterns using pattern recognition receptors (PRR) such as toll-like receptors (TLR) and nucleotide-binding oligomerization domain-containing (NOD) receptors (13,14). In particular, TLR4 recognizes lipopolysaccharide (LPS), which is shared by all Gram-negative bacteria, and TLR2 recognizes cell-wall components of Gram-positive bacteria. Upon activation of PRRs, intracellular signal transduction cascades are initiated resulting in activation of the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (13,15). Activation of NF-κB leads to the expression of chemokines, pro-inflammatory cytokines and anti-microbial peptides important for host defense (15). In addition, signaling via TLRs in intestinal epithelial cells leads to maintenance of the epithelial barrier, for example by increasing the strength of the tight junctions (16). To prevent excessive pro-inflammatory immune responses upon recognition of micro-organisms or constituents, innate immune cells acquire hyporesponsiveness to bacterial components. Hyporesponsiveness is a state of decreased reactivity to a certain stimulus after a previous encounter. An initial exposure to LPS reduces the sensitivity to a second exposure to LPS resulting in suppressed production of pro-inflammatory cytokines (17). This 'endotoxin tolerance' protects the host from endotoxic shock (18). Hyporesponsiveness is not a passive process, but a well-controlled response orchestrated to prevent excessive inflammation (17). Several mechanisms are responsible for innate immune cell hyporesponsiveness. First, PRR expression is downregulated after exposure to their ligands (19). TLR2 and TLR4 expression in the intestinal epithelium is low under homeostatic conditions, minimizing activation by luminal bacteria (16,20). In addition, PRRs are located strategically. For

example, TLR5, which recognizes flagellated bacteria, is preferentially expressed on the basolateral side of the polarized intestinal epithelial cells, where it recognizes translocated bacteria (16,20). In addition, a major mechanism for the acquisition of hyporesponsiveness to bacterial components is the expression of intracellular negative regulators. Negative regulators of the NF-κB pathway are induced by TLR ligands upon recognition of commensal bacteria, resulting in negative feedback (21). For example, Toll-interacting protein (TOLLIP) is expressed by intestinal epithelial cells in response to LPS and inhibits TLR2 and TLR4 signaling intracellularly (19,22,23). In addition, TNF alpha induced protein 3 (TNFAIP3, also known as A20) expression is induced by NF-κB signaling in intestinal epithelial cells and inhibits NF-κB activation (24). Moreover, the single Ig and TIR domain containing (SIGIRR) protein is an important regulator of colonic epithelial cell responsiveness (25,26). In monocytes and macrophages, IRAK-M expression is upregulated by TLR stimulation and negatively regulates TLR signaling, suppressing the production of pro-inflammatory cytokines including tumor necrosis factor alpha (TNF-α) (27). Thus, NF-κB inhibitors prevent pro-inflammatory immune responses in the absence of danger by keeping innate immune cells, including intestinal epithelial cells, in a hyporesponsive state.

Hyporesponsiveness of innate immune cells to the persistent commensals is also required to maintain an active tolerogenic adaptive immune response. Naïve $CD4^+$ T cells can differentiate into either regulatory T cells or inflammatory T cells after recognition of antigen and costimulation by antigen-presenting cells (APCs) in the mesenteric lymph nodes and gut-associated lymphoid tissue. The most important APCs are dendritic cells, but macrophages and B cells can also present antigens to naïve T cells. Although there is plasticity and heterogeneity, effector T cells can be subdivided based on their master transcription factor and production of cytokines into regulatory T cells, T helper 1 (Th1) cells, Th2 cells and Th17 cells (28). Regulatory T cells produce IL-10 and transforming growth factor beta (TGF-β) and suppress T-cell activity. Th1 cells protect against intracellular bacteria and viruses by activating macrophages via interferon gamma (IFN-γ), TNF-α and interleukin 6 (IL-6) production and help the activation of naïve B cells to become antibody producing plasma cells. Th2 cells also provide help to B cells and produce IL-4, IL-5 and IL-13, resulting in epithelial cell repair, recruitment and activation of eosinophils and the production of IgE, important for the elimination of extracellular parasites. Th17 cells produce IL-17A, IL-17F, IL-22, IL-21 and IL-6 and induce the production of chemokines by epithelial cells and stromal cells, resulting in neutrophil recruitment. Th17 cells are important in host defense against extracellular bacteria and fungi. IL-17A, the main cytokine produced by Th17 lymphocytes, has pro-inflammatory effects but can also protect the epithelial barrier by induction of the production of antimicrobial peptides and mucins by epithelial

cells (29). In addition, Th17 cells can promote repair of damaged intestinal epithelium during colitis together with regulatory T cells (30). Interestingly, both pro-inflammatory Th17 cells as well as tissue-protective Th17 cells exist (31). The differentiation of naïve T cells is driven by both extrinsic signals including cytokines and by intrinsic signals such as cellular metabolism (28). Under homeostatic conditions, intestinal epithelial cells promote a tolerogenic phenotype of resident lamina propria dendritic cells via the production of anti-inflammatory cytokines including TGF-β and thymic stromal lymphopoeitin (TSLP) (32-34). After encounter of harmless antigen and migration to gut-draining lymphoid tissues, these tolerogenic dendritic cells in turn promote the differentiation of regulatory T cells, but not Th1 and Th17 cells, via production of mucosal factors such as retinoic acid and TGF-β (35-37). This is associated with the production of IgA antibodies by plasma cells and prevents inflammatory responses by effector T cells (32,33). Indeed, in the intestine a large number of regulatory T cells differentiates in gut-draining lymphoid tissue, promoted by dendritic cells from the lamina propria (38). However, when intestinal epithelial cells are activated by invading micro-organisms, they produce pro-inflammatory cytokines and chemokines such as C-X-C motif chemokine ligand 8 (CXCL8; also known as IL-8), leading to the recruitment and activation of macrophages and neutrophils to the tissue which phagocytose and kill micro-organisms. In addition, antigen is no longer solely presented by tolerogenic dendritic cells but also by other subpopulations such as inflammatory monocyte-derived dendritic cells promoting the differentiation of inflammatory T cells, including Th1 and Th17 cells. After T cell activation, a significant number of T cells persist, known as memory cells. Memory T cells are long-lived and can quickly differentiate into effector cells when re-exposed to the same antigen, resulting in rapid and effective protective immunity to pathogens. Importantly, memory T cells need to be tightly regulated to prevent chronic pro-inflammatory responses to commensal bacteria, as occurs in inflammatory bowel disease (IBD).

In conclusion, intestinal epithelial cells form a barrier to the high load of luminal micro-organisms, exert protective innate immune functions and play a major role in controlling innate immune cell function and T cell responses, resulting in tolerance to commensal bacteria and elimination of invading micro-organisms. Key in this regulatory function of intestinal epithelial cells is the acquisition of hyporesponsiveness to the microbiota (Figure 1).

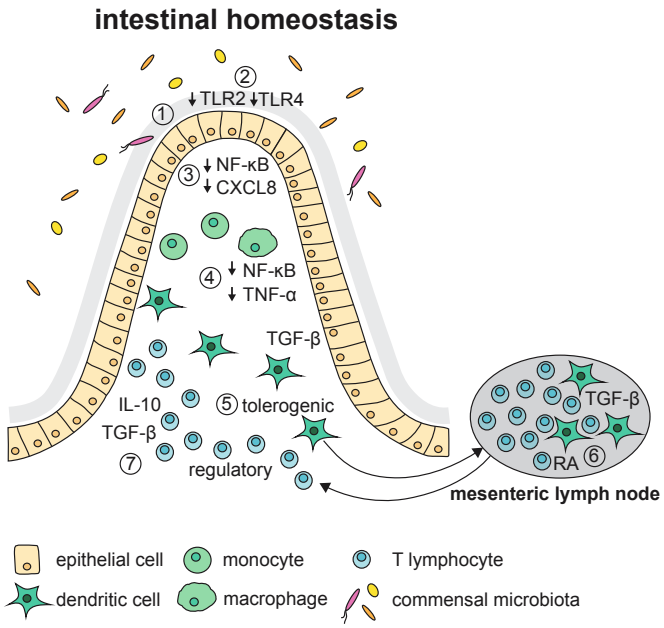


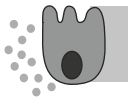
Figure 1: Innate immune cell hyporesponsiveness to commensal bacteria is crucial for intestinal homeostasis

(1) Intestinal epithelial cells form a physical barrier and produce mucus, which keeps most bacteria at a distance. (2) Intestinal epithelial cells are hyporesponsive to commensal bacteria via microbiota-induced downregulation of TLR2 and TLR4 expression. (3) Intestinal epithelial cells are hyporesponsive to commensal bacteria via microbiota-induced expression of intracellular negative regulators of NF-κB, resulting in suppressed production of chemokines including CXCL8. (4) Monocytes and macrophages are hyporesponsive to bacterial components via expression of intracellular negative regulators, resulting in suppressed production of pro-inflammatory cytokines including TNF-α. (5) Hyporesponsive intestinal epithelial cells produce cytokines including TGF-β, thereby inducing a tolerogenic phenotype of dendritic cells in the lamina propria. (6) Tolerogenic dendritic cells subsequently migrate to the mesenteric lymph nodes and induce the differentiation of regulatory T cells via production of anti-inflammatory cytokines including retinoic acid (RA) and TGF-β. (7) Regulatory T cells in turn migrate to the lamina propria where they produce IL-10 and TGF-β and inhibit effector T cell activity.

Secretory Leukocyte Protease Inhibitor (SLPI) drives intestinal epithelial hyporesponsiveness to commensal bacteria

Intestinal epithelial cells acquire hyporesponsiveness to TLR stimulation in the first weeks after birth, the period when the body is first colonized by bacteria (39,40). Previously, our laboratory has established that human buccal epithelial cells, which are easily accessible from the cheek, spontaneously produce CXCL8 and are responsive to TLR ligands directly after birth (40). In contrast, buccal epithelial cells from 2 to 6 months old infants and adults have lost responsiveness to TLR ligands. Repetitive

stimulation of buccal epithelial cells with TLR ligands leads to induction of Secretory Leukocyte Protease Inhibitor (SLPI), which inhibits NF-κB and subsequent CXCL8 production (40). Colonic SLPI expression is induced by colonization of the intestine of adult germ-free mice with a conventional microbiota and regulates epithelial hyporesponsiveness to the intestinal microbiota (40). Thus, SLPI is a regulator of the acquisition and maintenance of intestinal epithelial hyporesponsiveness. SLPI also regulates TLR signaling in monocytes and macrophages via inhibition of NF-κB, although endogenous expression of SLPI in these cell types has not been demonstrated (41-43).



SLPI inhibits TLR signaling in three ways: extracellular SLPI interferes with uptake of LPS (44), SLPI prevents degradation of the NF-κB inhibitor alpha (IκBα) in the cytoplasm (41), leading to suppression of TLR2 and TLR4 signaling (43), and SLPI directly prevents p65 binding to NF-κB by competing with p65 for NF-κB consensus-binding sites in the nucleus (42) (Figure 2). TOLLIP, TNFAIP3, SIGIRR and IRAK-M all interfere with TLR signaling in the cytoplasm (21). As hyporesponsiveness to TLR ligands in buccal and intestinal epithelial cells is associated with upregulation of SLPI, but low expression of TOLLIP, TNFAIP3 and SIGIRR, we anticipated that SLPI is crucial for intestinal epithelial hyporesponsiveness to commensal bacteria (Figure 2).

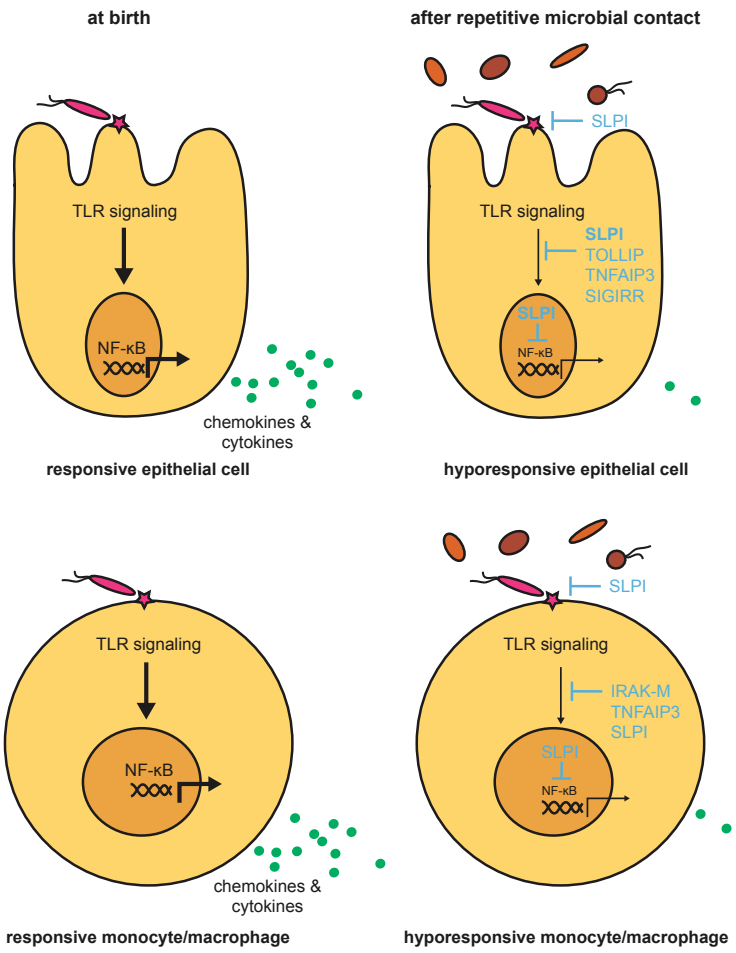
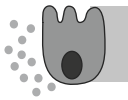


Figure 2: NF-κB inhibitors induce hyporesponsiveness to commensal bacteria in intestinal epithelial cells, monocytes and macrophages

Expression of NF-κB inhibitors is low immediately after birth, resulting in responsive intestinal epithelial cells, monocytes and macrophages. TLR signaling results in the activation of NF-κB, leading to the production of cytokines and chemokines. In contrast, after repetitive contact with commensal bacteria, intestinal epithelial cells acquire hyporesponsiveness via induction of NF-κB inhibitors, including SLPI, TOLLIP, TNFAIP3 and SIGIRR. In intestinal epithelial cells, SLPI expression is high compared to other NF-κB inhibitors. SLPI inhibits NF-κB activation both in the cytoplasm and nucleus. TOLLIP, TNFAIP3, SIGIRR and IRAK-M interfere with TLR signaling in the cytoplasm, preventing the translocation of NF-κB to the nucleus. In intestinal monocytes and macrophages, repetitive TLR stimulation results in the acquisition of hyporesponsiveness via induction of NF-κB inhibitors IRAK-M and TNFAIP3. In addition, exogenous SLPI has been shown to inhibit TLR signaling in monocytes and macrophages.

SLPI protects the intestinal mucosa against inflammation, tissue damage and infection

SLPI is an evolutionary conserved protein (45,46) produced by human epithelial cells, macrophages, neutrophils and mast cells (47-50). Many human epithelia express SLPI, including the respiratory epithelium and the skin epidermis (51-56). By inhibiting NF-κB activation, SLPI suppresses the production of pro-inflammatory cytokines by epithelial cells (40), monocytes (42), macrophages (57) and dendritic cells (58,59). Interestingly, SLPI has several functions other than inhibition of TLR signaling. Namely, SLPI inhibits proteases produced by immune cells including neutrophil elastase, thereby limiting tissue damage during inflammatory immune responses (55,60). Moreover, SLPI is capable of antibacterial, antifungal and antiviral activity (52,61-63). In particular, SLPI prevents entry of human immunodeficiency virus 1 (HIV-1) in host cells (64-66). Thus, SLPI prevents inappropriate immune responses, limits tissue damage caused by immune cells and protects the host against micro-organisms. It is therefore not surprising that SLPI expression is high at sites where contact with micro-organisms is concentrated, such as the lungs and the colon.



Inflammatory Bowel Disease (IBD) is characterized by an inappropriate pro-inflammatory immune response to commensal bacteria

IBD is a chronic inflammatory disease of the gastrointestinal tract. The two major clinical subtypes of IBD are Crohn's disease and ulcerative colitis, which are defined by clinical and pathological characteristics. Both patients with Crohn's disease and ulcerative colitis can suffer from abdominal pain, diarrhea and rectal bleeding. Crohn's disease is characterized by patchy transmural inflammation and can affect any part of the gastrointestinal tract. Granulomas, fissures, fistulas and perianal abscesses can arise in Crohn's disease patients. In ulcerative colitis, the colonic mucosa is affected by a diffuse and continuous inflammation, which predominantly involves the distal colon or the whole colon (pancolitis). Microscopically, actively affected intestinal tissue from both Crohn's disease patients and ulcerative colitis patients can show neutrophils in the crypt epithelium (cryptitis), neutrophils in the crypt lumina (crypt abscesses), erosions and ulcers. In addition, features of chronicity can be seen, including crypt distortion, crypt loss, crypt atrophy, basal plasmacytosis and Paneth cell metaplasia. However, no single histopathological feature is diagnostic of IBD or a subtype of IBD and the combination of clinical (including endoscopic) and histopathological findings is needed for optimal diagnostic accuracy and thereby optimal treatment (67).

The precise etiology of IBD is unknown, but genetic factors, the host immune system, the microbiota and environmental factors such as nutrition all contribute to the pathogenesis. Genetic variations predisposing to IBD are associated with mucosal

barrier function, regulation of immune responses and antimicrobial activity (68). The importance of environmental factors in the onset of the disease is clear from the fact that the prevalence of IBD is rising in countries which adopt a Western lifestyle (69). Environmental factors which are implicated in the pathogenesis of IBD include nutrition, infections and the use of antibiotics (68). These factors can transiently disrupt the mucosal barrier, stimulate immune responses or cause a disbalance in the microbiota composition (68). In IBD, the host immune system responds to the commensal microbiota in an aberrant manner (68). In both Crohn's disease and ulcerative colitis, an imbalance between pro-inflammatory $CD4^+$ T cells and regulatory T cells results in destructive inflammation (70). Pro-inflammatory T cells infiltrate the intestinal tissue and respond to the normally harmless microbiota. Several animal models have shown that without luminal bacteria, chronic intestinal inflammation does not develop (71-74). Thus, IBD patients exhibit loss of immunological tolerance to the microbiota (68). The chronicity of the disease is caused by reactivation of inflammatory memory T cells by the persisting luminal bacteria, leading to a relapsing-remitting disease course (75).

In Crohn's disease, Th1 cells have been considered to drive the disease, as high levels of IFN-γ and IL-12, which induce Th1 cell differentiation, have been found in intestinal tissue of Crohn's disease patients (76). However, more recently non-classical populations of Th17 cells which produce both Th17 and Th1 cytokines have been implicated in the disease (31,77). In agreement, high levels of IL-23 have been found in intestinal tissue of Crohn's disease patients (78). IL-23 is produced by dendritic cells and macrophages and induces proliferation of Th17 cells, which express the IL-23 receptor, and stimulates the production of IL-17A, IL-17F and IL-22 by Th17 cells (79). Especially the non-classical Th17 cells producing both Th17 and Th1 cytokines are highly sensitive to IL-23 stimulation (31). The number of pro-inflammatory Th17 cells is indeed enriched in intestinal tissue of Crohn's disease patients (31). Conversely, the IFN-γ driven responses are much less dominant in ulcerative colitis, which is thought to be driven by Th2 and Th17 lymphocytes. Cells from the lamina propria of patients with ulcerative colitis secrete increased amounts of IL-5 and IL-13, cytokines produced by Th2 cells (80,81). In addition, the expression of Th17 cytokines is increased in the mucosa of patients with ulcerative colitis (82,83). However, it remains to be established whether Th17 cells are pathogenic in IBD, as Th17 cells can both have pathogenic and protective functions. As the differentiation of Th17 lymphocytes is induced by contact with bacterial antigen, the increase in Th17 cells in IBD may be a secondary effect of bacterial translocation, for example due to a barrier defect. Cytokines including IL-23, produced by innate immune cells, regulate pathogenic Th17 cell function (79). In summary, IBD is characterized

by inflammatory T-cell responses to the commensal microbiota in a genetically susceptible host, which is likely to be triggered by environmental factors.

IBD is a heterogeneous disease in terms of course of disease and therapy responsiveness

IBD has a peak onset between 20 and 40 years of age, but in 10-25% of IBD patients the disease already starts during childhood (84,85). Early onset of IBD is associated with a more extensive and more aggressive disease (86). Interestingly, underlying monogenic disorders occur more frequently in IBD patients with early onset (87). These monogenic defects can inform on immunological mechanisms that play a role in the pathogenesis of IBD. In particular, genes involved in monogenic IBD patients share pathways with the susceptibility loci that have been associated with IBD (87-91). Therefore, in this thesis we focus on children when studying the underlying immune defects in IBD.

Both pediatric Crohn's disease patients and pediatric ulcerative colitis patients are heterogeneous groups with varying course of disease and therapy responsiveness. Children with IBD are treated with corticosteroids and other immunomodulators to induce and maintain remission (92,93). Anti-tumor necrosis factor alpha (TNF-α) biologicals including Infliximab are used in children with IBD refractory to conventional treatment (92,93). However, a substantial subgroup of IBD patients loses response to anti-TNF therapy and suffers from frequent exacerbations which require treatment intensification (94,95). Importantly, first-line treatment with Infliximab in children with moderate-to-severe Crohn's disease has recently been shown to be more effective in achieving and maintaining remission than conventional treatment (96). In particular, one year after the start of therapy first-line treatment with Infliximab was superior to conventional treatment in achieving remission without need for treatment escalation (96). However, some patients can achieve clinical remission without Infliximab and it is currently not possible to identify these patients on the basis of their clinical profile at diagnosis (96). Immunosuppressive medication can have serious side effects in children, such as severe infections and growth retardation. On the other hand, insufficient treatment of intestinal inflammation can also have a major negative impact on growth and development. Uncontrolled inflammation can lead to life-threatening complications such as strictures and perforations. Approximately 20% of children with ulcerative colitis needs a colectomy during childhood (93). For children with Crohn's disease the risk of having undergone an extensive intestinal resection by the age of 30 years is approximately 50% (97). Thus, effective therapy from diagnosis onwards is crucial and treatment should be tailored to patient subgroups in order to prevent over- and undertreatment. Therefore, predictors of disease course and treatment response are required.

Few predictive factors have been identified in pediatric IBD and a personalized approach to IBD treatment has not been realized yet (92,98). High levels of serum c-reactive protein (CRP) predict relapse and response to anti-TNF treatment in Crohn's disease patients (99). However, not all IBD patients with active disease have an elevated serum CRP (98). In addition, calprotectin concentrations in feces reflect the presence of neutrophils in the intestine and predict relapse in both CD and UC patients (99). However, both serum CRP and fecal calprotectin concentrations reflect ongoing inflammation at a certain timepoint instead of a subtype of IBD and may therefore not truly be predictive of course of disease and therapy responsiveness at diagnosis (98). In conclusion, there is a need for factors that can identify at diagnosis which IBD patients will benefit from a more aggressive therapeutic strategy.

IBD is a heterogeneous disease in terms of underlying immune defects

The underlying immune defects in patients with IBD are heterogeneous, as chronic intestinal inflammation can develop via many different mechanisms (68). Because each IBD subtype may have a unique course of disease and unique responses to various treatments, a 'one-size-fits-all' approach is unlikely to result in effective treatment for each individual patient (68,98). The identification of IBD subtypes based on distinct histopathological and cellular features is thought to aid precision targeting of treatment (100). In addition, in order to develop targeted treatments, it is crucial to identify subgroups of IBD patients based on biological processes (101).

One cause for the heterogeneity in course of disease and response to therapy in IBD patients may be the dual role for the innate immune response in the pathogenesis of IBD (102). On the one hand, hyperresponsiveness of epithelial cells, dendritic cells and macrophages cells to microbial contact leads to production of pro-inflammatory cytokines, resulting in pro-inflammatory T cell responses. Alternatively, insufficient antimicrobial defense can lead to infiltration and persistence of bacteria in the lamina propria, leading to the activation of pro-inflammatory T cells by persisting microbial antigens (101,102). Indeed, genetic defects in the innate immune system have been found in patients with Crohn's disease (68). A strong genetic risk factor for the development of Crohn's disease is a loss-of-function mutation in caspase recruitment domain family member 15 (CARD15), also known as nucleotide-binding oligomerization domain-containing protein 2 (NOD2), a member of the NOD-like receptor family of intracellular pattern recognition receptors (103). CARD15 is expressed by intestinal epithelial cells and is important for recognition of bacteria and subsequent antibacterial response via NF-κB activation (104). The importance of innate immune defects in the pathogenesis of IBD is also suggested by the observation that some patients with primary immune deficiencies develop IBD-like disease (87). For example,

chronic granulomatous disease (CGD) is an X-linked disease caused by genetic defects in nicotinamide adenine dinucleotide phosphate (NAPDH) oxidase, which is needed for the 'respiratory burst' to kill phagocytosed bacteria. CGD patients often develop IBD-like intestinal inflammation, most likely because bacteria are not eradicated from the lamina propria (87). Anti-TNF-α treatment predisposes CGD patients to severe infections, suggesting that aggressive suppression of immune responses can be dangerous in IBD patients with defects in antimicrobial responsiveness (105). Thus, IBD patients with innate immune deficiencies may benefit less from aggressive immunosuppression. However, methods to identify IBD patients with insufficiencies in their anti-microbial immune response are scarce.

Histopathological characterization of intestinal tissue from IBD patients has the potential to inform on immune disease subtypes

Despite the recent advances in understanding the heterogeneity in immune defects in IBD, classification of IBD patients based on their immune response in order to predict therapy response is not possible yet. Clinical characteristics alone are currently not able to reliably classify IBD patients to guide clinical decision making (98). We anticipated that the different immune processes in IBD could be evaluated in intestinal biopsies taken from IBD patients at diagnosis. In particular, immunohistochemistry could be used to assess the activation of innate immune cells or differentiate between different types of effector T cells. Moreover, expression of certain proteins by epithelial cells could be used to identify IBD patients with an insufficient anti-microbial immune response. It has been established that histopathological assessment of disease activity has clinical value, as persistent inflammation after treatment is related to clinical relapse (106). Therefore, mucosal healing is a treatment goal in clinical practice (107). However, few studies have assessed the value of histopathological characterization of IBD at diagnosis for prediction of therapy response. Some microscopic findings at diagnosis are known to predict a more severe disease phenotype. For example, in pediatric IBD, the presence of granulomas in diagnostic biopsies is associated with more severe disease and a shorter time to immune modulating treatment (108,109). In addition, gene expression signatures associated with therapy nonresponse were found to be associated with distinct histopathological features (100). This shows that histopathological characteristics at diagnosis can inform on course of disease and therapy responsiveness in IBD. However, the identification of immune subtypes of IBD by microscopy is largely unexplored.

Hypothesis: intestinal epithelial SLPI expression identifies IBD patients in which contact between epithelial cells and the microbiota is intensified

As intestinal epithelial SLPI expression increases after repetitive microbial contact and as SLPI regulates epithelial hyporesponsiveness to microbial signals, we hypothesized that intestinal SLPI expression increases when contact between epithelial cells and the microbiota is intensified, as occurs in IBD (Figure 3).

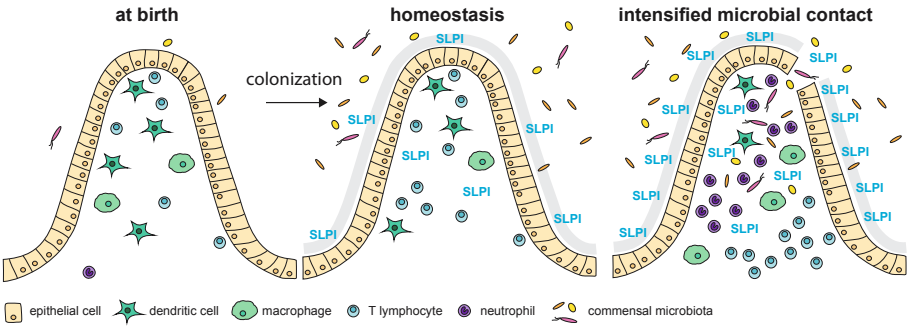


Figure 3: Intestinal epithelial SLPI expression is induced by microbial contact and we hypothesized that SLPI expression increases when epithelial-microbial contact is intensified

At birth, SLPI expression by intestinal epithelial cells is induced by the first contact between intestinal epithelial cells and bacteria (left image). During homeostasis, most commensal bacteria are separated from intestinal epithelial cells by the mucus layer and SLPI ensures that the epithelium remains hyporesponsive to harmless bacterial signals (middle image). We hypothesized that close contact between intestinal epithelial cells and the microbiota, for example due to a barrier defect, results in increased epithelial SLPI expression (right image). SLPI limits proinflammatory immune responses by inhibition of NF-κB signaling and protects against tissue damage by proteases such as neutrophil elastase.

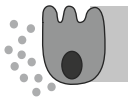
Therefore, we hypothesized that intestinal epithelial SLPI expression is especially high in IBD patients with a strong antimicrobial immune response, as occurs in patients with underlying innate immune defects. Thus, we anticipated that high SLPI expression in intestinal epithelial cells may identify IBD patients with a subtype of disease associated with a distinct clinical phenotype.

SLPI expression is increased in intestinal epithelial cancer cells

Recently, SLPI expression has been found to be increased in several types of cancer cells, including colorectal cancer (CRC) cells (110-114). Interestingly, overexpression of SLPI contributes to metastasis formation in mouse models for breast cancer, ovarian cancer and lung cancer (112,115,116). In human breast cancer and gastric cancer SLPI expression in has been associated with a poorer prognosis (112,117,118). However, the precise role of SLPI in cancer is unclear.

There is a need for prognostic factors in colorectal cancer (CRC)

CRC is the fourth leading cause of cancer-related mortality globally, and its incidence and mortality rates are still rising (119,120). Survival after resection of the primary tumor is highly variable in CRC, even among patients with similar clinical and pathological risk factors (121,122). Therefore, identification of prognostic factors is needed to enable selection of patients which will benefit from additional therapy after resection of the primary tumor.



The major cause of death in CRC patients is metastasis to distant organs and the liver is the most common site of distant metastasis in CRC (123). Resection of the affected liver tissue is the only curative treatment option for patients with CRC liver metastases (124). To optimize the prognosis after resection of CRC liver metastases, some patients receive additional treatment. As the prognosis after resection of CRC liver metastases is highly variable, even among patients with the same similar clinical and pathological risk factors (125), identification of patients which will benefit from additional therapy is needed. Thus, there is a need for prognostic factors both for CRC patients with localized disease and for CRC patients who underwent resection of liver metastases.

The identification of prognostic proteins involved in biological processes important in CRC has the potential to distinguish between clinicopathologically similar patients and thereby improve personalized treatment. The immune system plays a key role in the course of CRC tumor development (126) and one of the hallmarks of cancer is evading immune destruction (127). CRC cells are able to escape recognition and elimination by the immune system via multiple mechanisms, amongst which the secretion of immunosuppressive factors such as IL-10 and TGF-β by tumor cells (128). However, studies on the prognostic value of innate immune proteins in CRC are scarce (129).

Hypothesis: SLPI promotes CRC tumor growth and metastasis formation

It is unknown whether SLPI has a functional role in CRC. Interestingly, murine colon cancer cells overexpressing SLPI form tumors more rapidly than control cells after subcutaneous injection in mice (130). Based on SLPI's diverse functions and its possible role in other types of cancer, we hypothesized that SLPI promotes CRC tumor growth and metastasis formation in multiple ways. First, SLPI may inhibit NF-κB activation in cancer cells and immune cells, thereby suppressing chemokine production and recruitment of inflammatory cells such as macrophages and T cells to the tumor niche. This way, tumor SLPI may suppress host immunity against cancer. Second, inhibition of proteases by SLPI may prevent infiltration of immune cells in the tumor niche. In addition, SLPI has been shown to drive vascular mimicry, a process in which epithelial cancer cells differentiate into endothelial-like cells and form tubular structures, thereby

providing blood supply to hypoxic regions of the tumor (112). Moreover, SLPI may act as an anti-coagulant, further facilitating blood supply within the tumor (112).

AIMS AND OUTLINE OF THIS THESIS

The main aims of this thesis are:

1. to assess whether SLPI expression in the intestine of pediatric IBD patients is associated with an immune subtype of disease.
2. to assess whether SLPI expression in CRC predicts patient prognosis.
3. to gain insight on the biological role of SLPI in the healthy intestine, in the inflamed intestine of IBD patients, and in CRC tumor growth and metastasis formation.

Ad 1. We hypothesized that intestinal epithelial SLPI expression identifies a subtype of IBD. Therefore, we aimed to establish whether intestinal epithelial SLPI expression is heterogeneous in pediatric IBD patients and which immune processes are associated with high intestinal epithelial SLPI expression. In addition, we aimed to elucidate whether intestinal epithelial SLPI expression is associated with a clinical phenotype in IBD.

Ad 2. Well controlled studies on the prognostic value of SLPI expression in human CRC are lacking. Therefore, we aimed to establish whether SLPI expression in the primary tumor is associated with prognosis in stage II and stage III CRC patients. In addition, we aimed to establish whether SLPI expression in CRC liver metastases is associated with prognosis after resection of CRC liver metastases.

Ad 3. While a lot is known on the protective role of SLPI in the lungs (131), little is known on SLPI's role in the intestine. We anticipated that SLPI is important for intestinal homeostasis, as SLPI regulates the recruitment of immune cells via epithelial chemokines in response to microbial contact. Both in IBD and in CRC, SLPI may have diverse functions which may be beneficial or detrimental to the host. A better understanding of SLPI's role in IBD and in CRC could ultimately help to develop new therapeutic strategies.

SLPI maintains homeostasis at barrier tissues in several ways. In **chapter 2**, we give an overview of the diverse functions of SLPI and its role in the lungs, the skin, the gastrointestinal tract and the genitourinary tract. In addition, we review the literature on SLPI expression and SLPI function in different types of cancer. We also provide insight on the different mechanisms by which SLPI may contribute to cancer. Finally, we discuss a possible role of SLPI in the anti-cancer immune response.

IBD is a heterogenous disease and there is a strong need for the identification of distinct disease subtypes to improve therapy responses. We hypothesized that intestinal epithelial SLPI expression can identify IBD patients with a strong antimicrobial immune response. In **chapter 3**, we investigated colonic epithelial SLPI expression in two murine models in which increased microbial contact leads to colitis. In addition, we used intestinal biopsies from a well-characterized Rotterdam pediatric IBD cohort to detect epithelial SLPI expression in the inflamed colon of therapy-naive pediatric IBD patients. We compared clinical and histopathological features between patients with high and low colonic epithelial SLPI protein expression. By using immunohistochemistry to detect SLPI, we were able to asses epithelial SLPI protein expression independent of the composition of the biopsies. In addition, detailed scoring of histological disease activity enabled us to characterize the immune processes in patients with high epithelial SLPI expression. Moreover, we used RNA sequencing to compare gene expression profiles in biopsies from IBD patients with high and low epithelial colonic SLPI protein expression. Furthermore, we investigated the immune profile in the peripheral blood of patients with high and low epithelial colonic SLPI protein expression by quantification of multiple proteins in the plasma.

SLPI is not only expressed by epithelial cells. Previously, we detected expression of SLPI in cells in the intestinal lamina propria of mice (40). In **chapter 4**, we investigated SLPI expression and function in human monocytes. Upon migration from the bloodstream into the intestine, monocytes need to adapt to the local environment to maintain homeostasis or, in case of infection, to promote inflammation. During intestinal homeostasis, monocytes acquire hyporesponsiveness to microbial signals and thereby prevent inflammatory responses to the microbiota. We detected SLPI protein expression in intestinal lamina propria cells of healthy controls and patients with IBD. Using a human monocytic cell line, we assessed the function of endogenous SLPI production by human monocytes in response to microbial signals.

The prognostic value of SLPI expression in CRC is unknown. In chapter 4 and 5, we detected SLPI protein expression using immunohistochemistry in two Dutch cohorts of CRC patients. In **chapter 5**, we focus on CRC metastasized to the liver (stage IV). Using tissue microarrays from patients who underwent surgical resection of CRC liver metastases, we investigated the relationship between SLPI protein expression in liver metastases and overall survival. In addition, we compared SLPI protein expression in liver metastases to SLPI protein expression in the primary tumor and assessed the prognostic value of SLPI protein expression in the primary tumor. Finally, we evaluated whether high SLPI protein expression is a prognostic factor in patients with CRC liver metastases independent of established clinical risk factors.

In **chapter 6**, we questioned whether SLPI protein expression is associated with prognosis in CRC patients before distant metastases have established (stage II and stage III). We evaluated whether SLPI protein expression in the primary tumor is associated with disease recurrence in patients without lymph node metastases (stage II) or with lymph node metastases (stage III). As patients with micro-satellite instable (MSI) CRC have a better prognosis compared to patients with micro-satellite stable (MSS) CRC (132-134), we investigated the prognostic value of SLPI in patients with MSI tumors and in patients with MSS tumors separately. Furthermore, because a substantial group of stage III CRC patients in our cohort received adjuvant chemotherapy, we studied the prognostic value of SLPI separately for patients who did and who did not receive adjuvant chemotherapy. Finally, we assessed whether SLPI is a prognostic factor in localized CRC independent of established clinical risk factors.

Finally, in **chapter 7** we discuss the data described in this thesis in the context of the literature. In addition, we discuss which questions remain about the expression and function of SLPI in the healthy intestine, in IBD, and in CRC.

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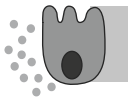

CHAPTER 2

Secretory Leukocyte Protease Inhibitor (SLPI) in mucosal tissues: protects against inflammation, but promotes cancer

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ABSTRACT

The immune system is continuously challenged with large quantities of exogenous antigens at the barriers between the external environment and internal human tissues. Antimicrobial activity is essential at these sites, though the immune responses must be tightly regulated to prevent tissue destruction by inflammation. Secretory Leukocyte Protease Inhibitor (SLPI) is an evolutionarily conserved, pleiotropic protein expressed at mucosal surfaces, mainly by epithelial cells. SLPI inhibits proteases, exerts antimicrobial activity and inhibits nuclear factor-kappa B (NF-κB)-mediated inflammatory gene transcription. SLPI maintains homeostasis at barrier tissues by preventing tissue destruction and regulating the threshold of inflammatory immune responses, while protecting the host from infection. However, excessive expression of SLPI in cancer cells may have detrimental consequences, as recent studies demonstrate that overexpression of SLPI increases the metastatic potential of epithelial tumors. Here, we review the varied functions of SLPI in the respiratory tract, skin, gastrointestinal tract and genitourinary tract, and then discuss the mechanisms by which SLPI may contribute to cancer.

1. SLPI STRUCTURE, FUNCTIONS AND REGULATION

1.1 Structure

SLPI is expressed in various human epithelia, including the salivary glands, the epidermis of the skin and the epithelia that line the respiratory, gastrointestinal and genitourinary tracts (1-6). SLPI initially had several tissue-specific names (see box 1); however, in 1988, these proteins were proven to be identical and encoded by a single gene in the human genome (7). The SLPI protein has a boomerang-like shape and contains two domains with similar architecture, with the polypeptide segments of each domain connected by four disulphide bridges (8). SLPI is a member of the whey-acidic protein (WAP) family, which all contain four-disulphide core domains (9). The gene encoding SLPI is evolutionarily conserved across birds, reptiles and mammals (10,11). Human and murine SLPI are 68% homologous at the genomic level and 60% homologous at the protein level (12-14), though the protease binding site and protease inhibitory capacity differ between species (15,16).

Box 1: What is SLPI?

- Low-molecular-weight (11,726 Daltons) 107-amino acid non-glycosylated protein (5,17,18)
- The human *SLPI* gene is located on chromosome 20q12 13.2 (9,14)
- Official name: Secretory Leukocyte Protease Inhibitor; alternative names: human seminal plasma inhibitor I, cervix uteri secretion inhibitor, bronchial secretory inhibitor, bronchial mucus inhibitor, bronchial leukocyte proteinase inhibitor, anti-leukoprotease
- Member of the WAP family, of which SLPI and Elafin are most-well studied (9)
- Produced by human epithelial cells, macrophages, neutrophils and mast cells (19-22)
- Produced by murine epithelial cells, macrophages, neutrophils, mast cells, germinal center B cells and innate lymphoid cells (The Immunological Genome Project; (23)
- Present in high concentrations in a variety of secretions, including saliva and nasal, bronchial, intestinal and cervical mucus (1,5,24,25)
- Can rapidly cross membranes and most likely does not need a receptor to enter cells (26)
- Identified functions include:
    - o Inhibits serine proteases, including neutrophil elastase, cathepsin G, chymotrypsin, trypsin and chymase (5,27-29); SLPI belongs to the chelonianin family of serine protease inhibitors (30)
    - o Inhibits TLR signaling by inhibiting uptake of lipopolysaccharide (LPS) and inhibiting NF-κB signaling (26,31,32)

- Antimicrobial protein: exerts antibacterial and antifungal activity and prevents HIV-1 transmission (2,33-35)
- Differentiation and survival factor for $CD34^+$ bone marrow hematopoietic progenitors (36)
- Anticoagulant (37,38)

1.2 Functions

1.2.1 Prevention of tissue destruction

SLPI strongly inhibits serine proteases, including neutrophil elastase (5,30). Leukocytes secrete proteases to facilitate their migration through the extracellular matrix of tissues and to kill phagocytosed microorganisms. Endogenous protease inhibitors counteract the action of these proteases to limit collateral tissue damage. Some protease inhibitors are produced by the liver ('systemic antiproteases'), while others are produced locally and upregulated by bacterial products and inflammatory cytokines ('alarm antiproteases') (39). SLPI and Elafin (also known as peptidase inhibitor 3 or skin-derived antileukoprotease) are two well-characterized human alarm antiproteases.

The region responsible for the protease inhibitory activity of SLPI is located on its C-terminal domain (8,28,40). SLPI is the major inhibitor of neutrophil elastase in the cytoplasm of neutrophils (21) and is the only elastase inhibitor that has been identified in saliva (41). SLPI retains its capacity to inhibit neutrophil elastase when cross-linked to fibronectin or elastin by tissue transglutaminase-2 and plasma factor XIIIa (42). In addition, SLPI inhibits the production of matrix metalloproteinases (MMPs) by monocytes (43) and can also prevent the formation of neutrophil extracellular traps (NET) (44). Overall, SLPI contributes to local tissue homeostasis by preventing damage by innate immune cells.

1.2.2 Regulation of inflammation

As well as counteracting the effects of proteases produced by innate immune cells, SLPI can also prevent the production of pro-inflammatory cytokines and the subsequent recruitment of immune cells. SLPI inhibits Toll-like receptor (TLR) signaling at three levels: extracellular SLPI interferes with the binding of LPS to soluble CD14 and the movement of LPS from CD14 into cell membranes (31); cytosolic SLPI prevents degradation of the NF-κB inhibitor alpha (IκBα) (45) to attenuate TLR2 and TLR4 signaling (32); and, in the nucleus, SLPI competes with p65 for NF-κB consensus-binding sites, and thereby directly prevents p65 binding (26). The ability of SLPI to inhibit NF-κB signaling is independent of the anti-protease activity of the protein, as amino acid substitutions in the C-terminal domain that disrupt the anti-protease

function of SLPI do not affect LPS-induced nitric oxide and tumor necrosis factor alpha (TNF-α) production by macrophages (46).

By inhibiting NF-κB signaling, SLPI shifts the balance of cellular cytokine production by suppressing production of proinflammatory cytokines by activated monocytes (TNF-α; IL-8) (26), dendritic cells (DCs; TNF-α; IL12p70) (47,48), macrophages (TNF-α; nitric oxide) (12) and epithelial cells (IL-8) (49). In turn, SLPI-induced modulation of monocyte function inhibits adaptive $CD4^+$ T helper cell proliferation and suppresses secretion of cytokines by T helper type 1 (Th1) cells, but does not alter $CD8^+$ cytotoxic T cell proliferation *in vitro* (50). SLPI also achieves selective $CD4^+$-Th1 suppression by increasing the production of IL-4, IL-6 and IL-10 by monocytes in the presence of T cell-derived IL-2 (50).

SLPI also indirectly regulates $CD4^+$ T cells *in vivo* via DCs. Specifically, in the lymph nodes draining the nasal mucosa, expression of SLPI in DCs attenuates the release of microbiota-induced IL-12p70, monocyte chemoattractant protein 1 (MCP1) and IL-6, thereby maintaining T cell-mediated mucosal tolerance to harmless proteins encountered at the densely colonized nasal mucosal surface (48). In addition, SLPI produced by tonsillar epithelial cells suppresses immunoglobulin class switching in activated B cells by inhibiting NF-κB signaling (51).

Overall, SLPI prevents the production of several pro-inflammatory cytokines and indirectly attenuates adaptive inflammatory immune responses, and thereby maintains balanced immune responses at mucosal barrier tissues.

1.2.3 Antimicrobial activity

SLPI also possesses broad-spectrum antibacterial, antifungal and antiviral properties (2,33,35,52). SLPI can directly kill *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Candida albicans* and *Aspergillus fumigatus* (2,33,35,52-54). In addition, SLPI is the only salivary protein that exerts activity against human immunodeficiency virus 1 (HIV-1) at physiological concentrations (55).

The N-terminal domain of SLPI possesses both antibacterial and antifungal activity, whereas the C-terminus exhibits low antibacterial activity (33,35). Therefore, the antibacterial activity of SLPI is most likely independent of its antiprotease activity. However, the mechanisms by which SLPI kills microorganisms are not entirely clear. SLPI can bind to *Escherichia coli* bacterial mRNA and DNA, which inhibits translation and arrests bacterial growth (56). In addition, the cationic nature of SLPI may allow the protein to attach to and destabilize the anionic cell membrane of bacteria (33,35). SLPI

can prevent transmission of HIV-1 via a mechanism independent of its antiprotease activity, as amino acid substitutions that reduce the protease inhibitor activity of SLPI do not affect its anti-HIV-1 activity (57). SLPI binds to human macrophages via annexin II, a cofactor involved in HIV-1 infection, and thereby disrupts the interaction between HIV-1 and macrophages (58). In addition, SLPI inhibits the interactions of the host membrane proteins phospholipid scramblase 1 and 4 with CD4, the main receptor for HIV-1 on T cells and macrophages (59).

Several microorganisms have evolved strategies to counteract the effects of SLPI. *Trichomonas vaginalis* secretes proteases that degrade SLPI (60) and *Streptococcus pyogenes* strains secrete streptococcal inhibitor of complement, which prevents bacterial cell killing induced by SLPI (54). *Pseudomonas aeruginosa* enhances the cleavage and inactivation of recombinant SLPI by neutrophil elastase (61). In addition, herpes simplex virus 1 and 2 are inhibited by SLPI and downregulate *SLPI* gene expression in human cervical epithelial cells (62).

1.2.4 Diverse functions of SLPI

Several recent studies uncovered novel functions for SLPI that still require further investigation. In particular, SLPI has been shown to inhibit apoptosis in human neutrophils and monocytes (63,64). Moreover, SLPI has been reported to be essential for the differentiation and survival of human $CD34^+$ bone marrow hematopoietic progenitors (36). However, detailed knowledge of the role of SLPI in myelopoiesis is lacking. In addition, SLPI may act as an anticoagulant. The plasma coagulation time is prolonged in *Slpi* knockout mice, despite normal thrombopoiesis (37), though it is not known whether SLPI acts as anticoagulant in humans.

1.3 Regulation of SLPI expression, production and secretion

SLPI is expressed by human epithelial cells (19,22), neutrophils (20,21), macrophages (65), mast cells (66) and fibroblasts (67). SLPI is secreted in saliva and mucus at high concentrations (1,5,24,25); the concentration of SLPI in saliva is 30-fold higher than in serum (41). Whether SLPI expression is regulated in a cell-type specific manner is unclear. In both epithelial and myeloid cells, *SLPI* mRNA is upregulated by a wide variety of TLR ligands and pattern recognition receptor ligands, such as Dectin-1 (12,47,49). In addition, TNF-α, interleukin-1 beta (IL-1β), transforming growth factor alpha (TGF-α), insulin-like growth factor 1 (IGF-1), progesterone and corticosteroids are all reported to increase *SLPI* mRNA expression in epithelial cells (22,52,68-70). Defensins increase the production of SLPI protein, but not mRNA expression, by epithelial cells (71). In macrophages, *SLPI* mRNA expression is upregulated by IL-10 and IL-6 and downregulated by interferon-γ (IFN-γ) (12,72,73). Interestingly, IFN-γ

restores the production of TNF-α and nitric oxide in response to LPS by SLPI-expressing macrophages, suggesting IFN-γ can overrule SLPI-induced tolerance to LPS (12). Furthermore, macrophages exposed to apoptotic cells secrete increased levels of SLPI, though the related mechanism is unknown (72). Secretion of SLPI by neutrophils can be enhanced by stimulation with phorbol 12-myristate 13-acetate (PMA), but not by LPS or granulocyte-macrophage colony-stimulating factor (GM-CSF) (21). Additionally, upregulation of SLPI in murine DCs occurs in the late stages after TLR stimulation and appears to be predominantly mediated by the MAPK pathway, rather than NF-κB signaling (47). However, it is not known whether SLPI expression is regulated by the MAPK signaling pathway in other cell types. The exact mechanisms responsible for *de novo* induction and/or upregulation of SLPI are poorly defined.

1.4 Inactivation of SLPI

Secreted SLPI can be inactivated by activated neutrophils via myeloperoxidase-catalyzed oxidation (74). In addition, MMP-9 destroys SLPI by cleaving both its N-terminal and C-terminal domains, which decreases the ability of SLPI to inhibit neutrophil elastase and attenuates the LPS-responsiveness of monocytes (75). Cleaved SLPI loses its capacity to suppress the production of MMP-9 by monocytes, suggesting that high production of MMP-9 can overrule SLPI (75). Similar observations have been made for the protease chymase, which cleaves SLPI (76) and is also inhibited by SLPI (29). Furthermore, cathepsin B, L and S can cleave SLPI, which inactivates its anti-neutrophil elastase activity (77). SLPI itself inhibits IFN-γ-induced cathepsin S production by macrophages via inhibition of NF-κB (78). Collectively, these data indicate a delicate balance exists between proteases and SLPI; this balance can shift towards inactivation of SLPI when the tissue is infiltrated by high numbers of innate immune cells, which may result in higher protease activity and tissue damage.

1.5 Conclusions

SLPI is produced by epithelial cells and immune cells, such as macrophages and neutrophils. Production of SLPI increases when these cells sense microorganisms through pattern recognition receptors or when stimulated by cytokines produced by innate immune cells. SLPI possesses a diverse range of functions, and intracellular and extracellular SLPI exert different effects (see box 2). Importantly, SLPI protects against excessive inflammatory immune responses at epithelial barriers. Moreover, secreted SLPI can be taken up by cells that do not express SLPI. Thus, the function of SLPI extends beyond the innate immune response.

One key function of SLPI is suppression of pro-inflammatory cytokine production, likely via a negative feedback mechanism, to prevent excessive inflammatory immune

responses after microbial contact. This suggestion is supported by the finding that *Slpi* knockout mice are more sensitive to a breach of mucosal tolerance to harmless antigens in the presence of LPS (48) and to LPS-induced endotoxin shock compared to wild-type littermates (79). However, SLPI is cleaved and inactivated in the presence of high numbers of activated innate immune cells. In addition, IFN-γ can inhibit SLPI production and possibly overrule SLPI-induced tolerance to LPS (12). These findings lead to the question of whether SLPI retains its tissue-protective capacity during chronic inflammation.

Slpi knockout mice do not exhibit overt abnormalities under specific pathogen-free (SPF) conditions, which is remarkable in view of the crucial roles of SLPI in immune regulation, tissue healing, antimicrobial defense, hematopoiesis and coagulation (79). These observations suggest that SLPI is only essential during tissue dysregulation, such as infection or chronic inflammation. As yet, no case reports of individuals with SLPI deficiencies have been described. However, the production of SLPI varies among healthy individuals (52), and it is unclear whether this variation is intrinsic or depends on environmental factors, such as microbial colonization.

Box 2: SLPI exerts varied functions, depending on the cellular location

- Extracellular:
  - Inhibits proteases (summarized in (30))
  - Inhibits uptake of LPS by macrophages (31)
  - Kills bacteria and fungi (33,35)
- Cytoplasmic:
  - Inhibits proteases
  - Prevents degradation of IκBα and IRAK (32)
  - Inhibits entry of HIV-1 into host cells (59)
- Nuclear:
  - Directly blocks NF-κB binding sites in the nucleus (26)

2 SLPI AT DIFFERENT BARRIER SITES

The functions of SLPI have been studied in different tissues, in the context of various human diseases and using multiple animal models. Here, we discuss the roles of SLPI at the main barriers between the external environment and the body - the respiratory tract, skin, gastrointestinal tract and genitourinary tract (see table 1 and figure 1) - focusing on tissue homeostasis and chronic inflammation.

2.1 SLPI in the respiratory tract

SLPI is produced in the lungs by bronchial epithelial cells, alveolar macrophages and neutrophils (4,21,65,80). SLPI isolated from human bronchial secretions is a strong inhibitor of neutrophil elastase and accounts for the majority of the total molar concentration of neutrophil protease inhibitors in bronchoalveolar lavage (BAL) fluid (81-83). SLPI is responsible for the majority of the anti-elastase activity in the upper respiratory tract; in contrast, in the peripheral airspaces, α_1-antitrypsin (A1AT) is more abundant and most SLPI protein is inactive (84-86). *SLPI* mRNA is expressed at 30-fold higher levels in human airway submucosal glands compared to the superficial epithelium (87). Neutrophil elastase and defensins increase SLPI production in airway epithelial cells (88,89), whereas TGF-β inhibits SLPI production (90,91). IL-1β and TNF-α upregulate SLPI expression in alveolar epithelial cells (22). SLPI is also present and exclusively associated with elastin fibers in lung connective tissue, suggesting that SLPI protects these fibers against degradation by elastase (92).

The roles of SLPI in disease have been studied most extensively in the lungs. An imbalance between proteases and protease inhibitors contributes to lung destruction in chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) (93). Chemotactic peptides are released when proteases break down collagen and elastin, resulting in accumulation of neutrophils and increased production of proteases (53,94,95). In addition, neutrophil elastase stimulates respiratory epithelial cells to produce IL-8, a strong chemoattractant for neutrophils (96,97). SLPI limits neutrophil-induced lung destruction by inhibiting neutrophil elastase and attenuating IL-8 production in bronchial epithelial cells (98,99). However, as neutrophil elastase also inactivates SLPI, this protective effect can be lost when high levels of neutrophil elastase are present (71,86,100). The sputum of patients with COPD contains higher levels of SLPI during acute exacerbations compared to patients with stable COPD or healthy individuals (101). However, the BAL fluid of patients with COPD also contains higher levels of IFN-γ and cathepsins compared to healthy controls; which results in cleavage and inactivation of SLPI in the epithelial lining fluid of patients with emphysema, but not in healthy controls (77,78). Thus, the insufficient amounts of SLPI in the sputum of patients with COPD fail to control the activity of neutrophil proteases, and this balance is disturbed even more during exacerbations (102). Conventional treatment for severe acute COPD exacerbations results in an increase in the sputum SLPI concentration within 48 hours, suggesting recovery of this balance (103).

The concentrations of SLPI in BAL fluid are not different between patients with CF and healthy controls (104). However, despite containing normal concentrations of SLPI, the lung epithelial lining fluid of children with CF contains higher concentrations of active

neutrophil elastase (105). In addition, patients with CF are often chronically infected with *Pseudomonas aeruginosa,* a bacterium that produces an elastase (106,107). SLPI exerts antibacterial activity against *Pseudomonas aeruginosa* (2), but both neutrophil elastase and *Pseudomonas aeruginosa* are able to cleave and inactivate SLPI (61,86). Indeed, patients with CF infected with *Pseudomonas aeruginosa* have lower SLPI levels and higher levels of neutrophil elastase in their BAL fluid compared to *Pseudomonas aeruginosa*-negative patients with CF (61,104). Moreover, SLPI is cleaved in the lower airway secretions of patients with non-CF bronchiectasis (75). In addition, in patients with allergic rhinitis and allergic asthma, the ratio of cleaved SLPI to total SLPI positively correlates with the concentration of chymase in nasal lavage fluid (108). Taken together, this evidence indicates that the levels of active SLPI are insufficient to counteract the elevated production of proteases in chronic lung diseases.

These observations suggest that administration of SLPI could be beneficial in chronic lung diseases. Recombinant SLPI has been shown to inhibit IL-8-induced neutrophil chemotaxis and decrease degranulation of MMP-9, cathelicidin and myeloperoxidase by neutrophils (109). Interestingly, higher concentrations of recombinant SLPI are needed to inhibit the chemotaxis of neutrophils isolated from patients with COPD or CF compared to neutrophils from healthy subjects (109). Hypersecretion of mucus is a feature of both COPD and CF, and can be counteracted by recombinant SLPI *in vitro* (110). Moreover, recombinant SLPI ameliorates the severity of disease in several animal models of COPD (111-114). Interestingly, intratracheal administration of recombinant SLPI reduced lung damage in an IgG immune complex-induced model of acute alveolitis in rats, at least partially by inhibiting epithelial NF-κB signaling (114,115). Inhibition of SLPI using a blocking antibody significantly increased lung injury in the same model, and was associated with abundant neutrophil accumulation (116). In addition, overexpression of *Slpi* reduced the signs of asthma in two mouse models of allergic asthma (117,118). Conversely, *Slpi* knockout mice developed more severe allergic asthma (118). Aerosolization of recombinant SLPI was proven to inhibit neutrophil elastase activity in the airway epithelial lining fluid of healthy humans (119). Aerosolized recombinant SLPI also reduced neutrophil elastase activity in airway epithelial lining fluid and reduced *IL8* mRNA expression in the bronchial epithelial cells of patients with CF (119-121). However, aerosolized SLPI does not reach the poorly ventilated areas of the lungs in patients with CF or emphysema (122). Moreover, hypoxia downregulates SLPI expression in bronchial epithelial cells (123). More stable variants of SLPI that are less susceptible to neutrophil elastase degradation and oxidation—but still bind to LPS and inhibit NF-κB signaling—have been developed (124,125).

In conclusion, SLPI production maintains respiratory tract homeostasis by inhibiting proteases, suppressing chemokine production and exerting antibacterial activity. However, SLPI activity is insufficient to counteract the excessive production of proteases in chronic lung diseases.

2.2 SLPI in the skin

SLPI is expressed and upregulated by calcium in human keratinocytes (2,126). The role of SLPI in the skin has been studied in the context of wound healing. Sterile wounding of human skin induces SLPI expression in keratinocytes via activation of the epidermal growth factor receptor (EGFR) by the growth factors IGF-1 and TGF-α (127,128). SLPI is essential for cutaneous wound healing in mice (129,130). *Slpi* knockout mice suffer delayed wound healing associated with increased elastase activity, accumulation of neutrophils and monocytes, and increased activation of TGF-β in the wounded skin (129). Mice deficient in Slpi due to a deficiency in natural resistance-associated macrophage protein 1 (Nramp1) also suffer delayed wound healing associated with overexpression of TGF-β (130). Interestingly, neutralization of TGF-β does not reverse the delayed wound healing in *Slpi* knockout mice as effectively as exogenous SLPI, suggesting that SLPI promotes wound healing via additional mechanisms beyond suppression of TGF-β alone (129). Indeed, SLPI also promotes wound healing by preventing the conversion of progranulin to granulin peptides by neutrophil elastase. Progranulin is an epithelial growth factor that blocks the activation of neutrophils by TNF-α. In contrast, granulin peptides induce IL-8 secretion and thereby attract neutrophils. Crucially, progranulin restores wound healing in *Slpi* knockout mice (131). Whether the antimicrobial activity of SLPI also promotes wound healing is unclear. SLPI is able to kill several microorganisms that can infect the skin (2); however, the relevance of the antimicrobial activity of SLPI in the skin is unknown. SLPI may also prevent scarring after wound healing, as it inhibits excessive contraction of collagen gel by human fibroblasts derived from hypertrophic scar tissue or keloid tissue (132).

SLPI also plays a role in unwounded skin. SLPI inhibits shedding from the upper epidermis by inhibiting the keratinocyte product kallikrein-related peptidase 7 (KLK7), which cleaves desmosomes in the stratum corneum (133,134). SLPI is the major endogenous inhibitor of human KLK7 (134). Indeed, overexpression of SLPI in the skin results in thickening of the stratum corneum in mice (135,136).

The function of SLPI in skin diseases is not well-studied. SLPI expression is elevated in the lesioned epidermis of patients with psoriasis (137), possibly due to increased production of the growth factors IGF-1 and TGF-α (128,138). Interestingly, SLPI colocalizes with neutrophil elastase as a component of NETs in psoriatic skin (139).

SLPI, together with neutrophil elastase and DNA, stimulates type I IFN production by plasmacytoid DCs (139), which play a key role in the pathogenesis of psoriasis (140).

In summary, SLPI is essential for murine wound healing, partly by suppressing immune cell infiltration (129). Whether SLPI also plays a role in human wound healing is unknown, though the increased expression of the protein in keratinocytes in skin wounds suggests SLPI may be involved in wound healing in humans. In addition, SLPI regulates shedding of the human and murine epidermis (134). SLPI may also contribute to the development of psoriasis in humans (139). However, the role of SLPI in chronic skin diseases is largely unknown.

2.3 SLPI in the gastrointestinal tract

SLPI is expressed by epithelial cells in the healthy intestine, in both the Paneth cells at the base of the crypts and goblet cells scattered throughout the epithelium (1). SLPI expression is induced in intestinal epithelial cells by microbial contact, as colonization of germ-free mice results in colonic SLPI expression (49). Secretion of SLPI by intestinal epithelial cells, mainly from the apical side, is stimulated by TNF-α, IL-1β and the protein kinase C pathway (52). SLPI protein levels in intestinal lavage fluid vary widely among healthy individuals (52). SLPI in intestinal fluid is likely to be locally produced, as salivary SLPI is rapidly degraded in the stomach and duodenum (52,141). As the concentration of SLPI in luminal fluid is relatively low, the SLPI secreted by epithelial cells is likely to exert its effects locally in the crypt or at the surface of the epithelium (52). In mice, SLPI protein is expressed at higher levels in the colonic epithelium compared to the small intestinal epithelium (49). In contrast, higher SLPI protein expression is observed in the lamina propria cells of the small intestine than those of the colon (49). It is not known whether the higher expression of SLPI in the colonic epithelium is the result of more frequent contact between bacteria and epithelial cells in the colon or due to contact with certain colonic bacteria. SLPI expression increases in the colonic epithelial cells of wildtype mice during induction of acute murine colitis by dextran sodium sulphate (DSS) (142,143). Oral delivery of lactic acid bacteria expressing murine SLPI or human Elafin ameliorates DSS-colitis in wildtype mice (144). In thymic stromal lymphopoietin (TSLP)-deficient mice, SLPI expression decreases during DSS-induced colitis (142). Interestingly, these mice fail to recover from DSS-induced colitis, due to excessive colonic neutrophil elastase activity. Administration of recombinant SLPI reduces the mortality rate of mice with DSS-induced colitis, suggesting that SLPI reduces protease-induced injury in the inflamed intestine (142). Indeed, SLPI protects intestinal epithelial cells against destruction by neutrophil elastase and trypsin *in vitro* (52). These findings lead to the question of whether SLPI protects against protease-induced tissue damage in inflammatory bowel disease (IBD). The levels of both neutrophil elastase and SLPI

are increased in the intestine of patients with IBD, though it is unclear whether these elevated SLPI concentrations are sufficient to protect against the increased elastase activity (145).

Concomitantly, SLPI plays an anti-inflammatory role during intestinal immune responses. SLPI inhibits NF-κB signaling in intestinal epithelial cells to reduce epithelial chemokine production in response to microbial triggers, and thus prevents continuous leukocyte infiltration at densely colonized mucosal surfaces (49). This mechanism is acquired directly after birth in the human buccal epithelium, when repetitive microbial interactions induce SLPI and impose acquired hyporesponsiveness within the epithelial cells to microbial signals from the oral microbiota (49). Similarly, SLPI ensures that the mucosal immune system maintains an adaptive tolerogenic response to harmless protein antigens, despite barrier sites being continually challenged with microbial products. Selective expression of SLPI in the DCs in mucosa-draining lymph nodes locally attenuates DC activation, in particular IL12p70 production, in response to LPS (48). Thus, SLPI maintains the regulatory T cell response to mucosally encountered harmless proteins (48). Indeed, *Slpi* knockout mice fail to acquire regulatory T cell-mediated mucosal tolerance to nasal administration of ovalbumin in the presence of low-dose LPS, while tolerance is unaffected in wildtype mice (48,146).

Overall, SLPI protects intestinal tissues from degradation by proteases and against excessive inflammation by attenuating the sensitivity of both intestinal epithelial cells and DCs in mucosa-draining lymph nodes to microbial triggers (48,49). SLPI is able to kill the intestinal pathogen *Salmonella typhimurium* (52), though the possibility that SLPI shapes the intestinal microbial composition has not been explored. SLPI promotes tissue repair in the oral mucosa via mechanisms similar to those found in the skin (147). However, it is unknown whether SLPI also contributes to healing of the intestinal mucosa.

Notably, *Slpi* knockout mice do not develop spontaneous intestinal inflammation under SPF conditions (79). We anticipate that *Slpi* knockout mice may develop intestinal disease after colonization with *Helicobacter hepaticus*, a bacterium that induces colitis in mice with immune regulation defects. In addition, we hypothesize that *Slpi* knockout mice may be more sensitive to infection with *Citrobacter rodentium*, a pathogen that models human infection with *Escherichia coli*. Another notable observation is that decreased SLPI expression does not result in impaired neutrophil infiltration during DSS-induced colitis (142), suggesting that SLPI may not play an essential role in granulopoiesis in mice.

2.4 SLPI in the genitourinary tract

SLPI is expressed by epithelial cells in the female genitourinary tract and secreted into vaginal fluid and cervical mucus (25,148-150). SLPI is also expressed in epithelial cells in the prostate, seminal vesicles and epididymis and is secreted in seminal plasma (151). However, the function of SLPI in the male genitourinary tract is unknown.

The antimicrobial capacity of SLPI in the female genitourinary tract has been studied. Perinatal HIV-1 transmission rates are lower among woman with high levels of SLPI in vaginal fluid (149), but whether this effect is due to SLPI inhibiting HIV-1 entering host cells has not been formally demonstrated. The levels of SLPI in vaginal fluid are decreased in women with *Trichomonas vaginalis*, *Neisseria gonorrhoeae* or *Chlamydia trachomatis* infections of the lower genital tract (152). Moreover, low levels of SLPI in vaginal swab specimens from healthy women have been associated with a higher *Trichomonas vaginalis* load, high vaginal pH and vaginal leukocytosis (153). These findings suggest either downregulation of SLPI by these bacteria or pre-existing low levels of SLPI impair bacterial cell killing. Indeed, SLPI exerts bactericidal activity against *Neisseria gonorrhoeae,* and expression of SLPI by reproductive tract epithelial cells *in vitro* is not altered by infection with *Neisseria gonorrhoeae* (150). However, SLPI can be inactivated by cysteine proteases produced by *Trichomonas vaginalis* (60). In contrast, *Chlamydia trachomatis* upregulates production of SLPI in cervical epithelial cells (154). In conclusion, these data indicate a role for SLPI in antimicrobial defense in vaginal fluid. However, establishment of a lower genital tract infection may lead to lower SLPI levels.

2.5 Comparison of the function of SLPI at different barrier sites

SLPI is produced and secreted at barriers by epithelial cells and infiltrating immune cells, such as neutrophils and monocytes. SLPI is upregulated by TLR ligands and pro-inflammatory cytokines in both intestinal and lung epithelial cells. In the respiratory tract, SLPI is an important inhibitor of proteases, and is inactivated in chronic lung diseases characterized by high protease activity. Local administration of recombinant SLPI holds promise for the treatment of chronic lung diseases characterized by excessive protease production. In the gastrointestinal tract, SLPI may also protect tissues from protease-induced damage during inflammation, though this has not been formally demonstrated. Importantly, SLPI regulates epithelial responsiveness to microbial signals in the oral and intestinal mucosa. Whether these mechanisms also occur in respiratory and genital epithelial cells has not yet been explored. In the skin and oral mucosa, SLPI promotes tissue repair after wounding, by both inhibiting proteases and exerting anti-inflammatory activity. Finally, SLPI possesses broad

antimicrobial activity; however, the relevance of this function has so far only been established in HIV-1 transmission.

In conclusion, the varied functions of SLPI have not been systematically studied in all tissues (see table 1 and figure 1). Therefore, it remains unclear whether SLPI exerts a unique role in each tissue type, or whether the inflammatory process determines the function of SLPI in specific tissues. We anticipate that SLPI exerts crucial immune regulatory functions in the respiratory tract and genitourinary tract, as in the gastrointestinal tract. In addition, SLPI may promote mucosal wound healing in other barrier tissues comparable to its role in cutaneous wound healing. Further studies of the diverse functions of SLPI in individual tissues will provide a more complete understanding of the roles of this pleiotropic protein in mucosal barriers and the skin.

Table 1: Main functions of SLPI at various barrier sites based on human and animal studies

	Tissue repair	**Anti-inflammatory activity**	**Anti-microbial activity**
Respiratory tract	Prevents tissue destruction by inhibiting proteases (*in vitro* and *in vivo*) (81,82,119)	Reduces alveolitis via inhibition of NF-κB (*in vivo*) (114,115); reduces allergic asthma (*in vivo*) (117,118)	Kills lung pathogens (*in vitro)* (2)
Skin	Promotes wound healing via protease inhibition and suppression of TGF-β (*in vivo*) (129)	Promotes wound healing via suppression of neutrophil recruitment (*in vivo*) (129,131)	Kills skin commensals and pathogens (*in vitro*) (2)
Gastrointestinal tract	Promotes oral wound healing via protease inhibition (*in vivo*) (147); ameliorates intestinal tissue damage caused by proteases (*in vitro* and *in vivo*) (52,142,144)	Suppresses epithelial chemokine production in response to microbial antigens via NF-κB inhibition (*in vitro* and *in vivo*) *(49)*; maintains tolerance to harmless protein antigens encountered at microbiota-rich mucosal sites (*in vivo*) *(48)*	Kills intestinal pathogens (*in vitro*) (33,52)
Genitourinary tract	Unknown	Unknown	Prevents HIV-1 infection (*in vitro*) (34); kills bacteria that cause lower urinary tract infections (*in vitro*) (2,150)

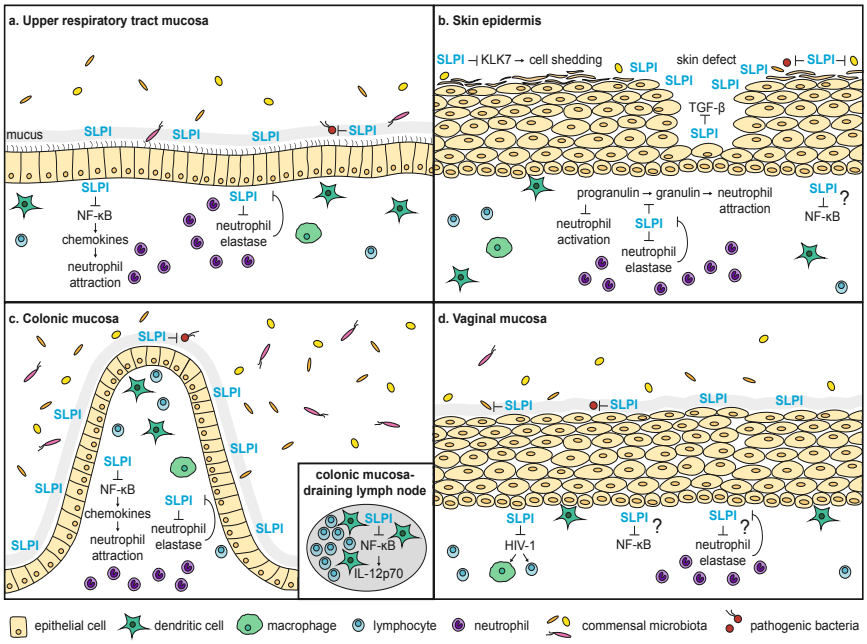

Figure 1: SLPI maintains tissue homeostasis at various barrier tissues

(a) In the mucosa of the upper respiratory tract, SLPI prevents tissue destruction by neutrophils via inhibition of neutrophil elastase. In turn, neutrophil elastase inactivates SLPI. In addition, SLPI inhibits the activation of epithelial NF-κB and thereby suppresses chemokine production and neutrophil attraction. SLPI also possibly kills pathogenic bacteria in the lung. (b) In the skin, SLPI inhibits keratinocyte shedding in the upper epidermis by inhibiting KLK7. SLPI is upregulated in keratinocytes after wounding. SLPI contributes to wound healing by inhibiting neutrophil elastase, inhibiting TGF-β and preventing conversion of the epithelial growth factor progranulin to granulin. Granulin promotes attraction of neutrophils by inducing production of chemokines by epithelial cells. Furthermore, SLPI can kill both skin commensals and pathogens. Whether SLPI inhibits NF-κB in keratinocytes is unclear. (c) In the colonic mucosa, SLPI suppresses epithelial cell production of chemokines by inhibiting activation of NF-κB in response to microbial contact. SLPI also inhibits neutrophil elastase in intestinal tissue. In addition, SLPI can kill intestinal pathogens. In mucosa-draining lymph nodes, SLPI prevents activation of DCs by inhibiting IL-12p70 production in response to microbial signals. (d) In the vaginal mucosa, SLPI prevents the entry of HIV-1 into macrophages and T cells and can kill bacteria and fungi. Whether SLPI inhibits NF-κB in the vaginal epithelium is unknown. Finally, inhibition of proteases by SLPI has not been studied in the female reproductive tract.

3. SLPI IN CANCER

SLPI is upregulated in several types of carcinoma, though its role in cancer has not been completely elucidated. SLPI has been suggested to be involved in cancer via multiple mechanisms (see box 3). Protease inhibitors were initially anticipated to protect against cancer, as degradation of the ECM is necessary for tumor growth and

invasion. However, recent studies showed that SLPI contributes to tumor growth and metastasis; though, counterintuitively, some functions of SLPI may actually protect against cancer.

Box 3: Mechanisms by which SLPI may influence tumor growth and metastasis

Mechanisms that may prevent cancer:

- Inhibits proteases, resulting in reduced ECM degradation and decreased cell invasion (155,156)
- Induces apoptosis, by inhibiting NF-κB (157) or downregulating E-cadherin and relocating β-catenin (158,159)
- Prevents virus-induced cancers, such as HPV-induced squamous cancer (160,161) and EBV-induced nasopharyngeal carcinoma (162)
- Prevents liver metastases by inhibiting expression of TNF-α and vascular adhesion receptor E-selectin in the liver (163)

Possible cancer-promoting mechanisms:

- Vascular mimicry: promotes formation of vessel-like structures by tumor cells (38)
- Anticoagulative activity (38)
- Promotes cell invasion via induction of MMP-2 and MMP-9 (156,159,164,165)
- Protects the epithelial growth factor progranulin by preventing elastase-mediated degradation or acting as a survival chaperone (166-168)
- Promotes cell invasion by reducing production of the antiangiogenic factor Endostatin via inhibiting elastase (155)
- Inhibits apoptosis by inducing cyclin D1 (169)

3.1 SLPI in breast cancer

High *SLPI* mRNA expression correlates with shorter overall survival and shorter distant metastasis-free survival in patients with triple-negative breast cancer (164). Moreover, in patients with aggressive subtypes of breast cancer, *SLPI mRNA* is expressed at higher levels in the primary tumors of patients with lung metastases than patients without metastases (38).

Importantly, SLPI has been shown to be a driver of metastasis in a mouse model of breast cancer heterogeneity (38); in this model, orthotopically injected mouse breast cancer clones expressing *Slpi* enter the vasculature more efficiently (38). SLPI is thought to participate in intravasation, as the metastatic potential was abrogated when the *Slpi* clones were administered via intracardiac injection. Two mechanisms have been proposed to explain the metastasis-promoting role of SLPI. First, SLPI programs

tumor cells for vascular mimicry, a process in which tumor cells differentiate into CD31-negative endothelial-like cells and form tubular structures that carry blood to the hypoxic regions of the tumor (38). Secondly, the metastatic potential of SLPI is reduced in warfarin-treated mice, suggesting that the anticoagulant function of SLPI contributes to its pro-metastatic role (38).

Other researchers also found a metastasis-promoting role for SLPI in mouse models of mammary carcinoma, and several mechanisms have been suggested to explain this observation (164,170). SLPI interacts with the retinoblastoma tumor suppressor protein, which results in increased translation of target genes such as *MMP2* and *MMP9* (164). In addition, another study found well-developed sinusoidal vessels surrounded tumor cells overexpressing *Slpi;* the authors suggested that SLPI acts via an invasion-independent metastasis pathway, in which tumor nests enveloped by sinusoidal vessels are released in the bloodstream (170).

In contrast, SLPI induces apoptosis in breast cancer cells *in vitro*, possibly by downregulating E-cadherin to lead to nuclear localization of β-catenin (158,171). Another study reported that SLPI reduces the growth of murine breast cancer tumors *in vivo* (171).

In summary, multiple studies have shown that SLPI promotes metastasis in mouse models of mammary carcinoma, though the precise mechanisms remain unclear.

3.2 SLPI in ovarian cancer

SLPI expression is increased in ovarian cancer compared to normal ovaries (172,173). In addition, serum SLPI levels are elevated in patients with early and late stage epithelial ovarian cancer, and serum SLPI can discriminate between patients with malignant and benign ovarian tumors (174-176).

Multiple studies suggest SLPI exerts a pro-metastatic role in ovarian cancer. SLPI promotes proliferation and prevents apoptosis in human ovarian cancer cell lines *in vitro*, independent of its protease inhibitory activity (166,168). In contrast, other researchers reported that SLPI inhibits cell proliferation, increases apoptosis and decreases the invasive ability of human ovarian cancer cell lines *in vitro* (177). However, in an orthotopic mouse model of ovarian cancer, overexpression of SLPI led to more metastases, again independent of the protease inhibition activity of SLPI (156,168).

One possible mechanism that may explain the tumor-promoting effects of SLPI is its ability to protect the survival factor progranulin, partly via inhibition of elastase-

induced degradation (166) or independently of protease inhibition (168). Another possibility is that SLPI increases MMP-9 production, independently of its protease inhibitory function (156). Overall, the evidence indicates SLPI is upregulated in ovarian cancer and acts as a pro-tumorigenic factor in mouse models, possibly by protecting progranulin and inducing MMP-9.

3.3 SLPI in squamous cell carcinoma of the head and neck (HNSCC)

In contrast to most other carcinomas, SLPI is expressed at low levels in HNSCC. SLPI is downregulated in oral premalignant lesions and oral squamous cell cancer compared to normal oral epithelium (157,178). In addition, high SLPI expression in oral premalignant lesions was associated with lower histological grade (179). Surprisingly, expression of SLPI in oral squamous cell carcinoma is associated with a better prognosis (180). Moreover, SLPI is expressed at higher levels in the HNSCC tumors of patients without lymph node metastases compared to patients with lymph node metastases (181). In contrast, expression of SLPI in tonsillar squamous cell carcinoma is associated with shorter overall survival (182).

SLPI is thought to play a role in HNSCC via multiple mechanisms, which makes it difficult to dissect the net effect of SLPI. Some *in vitro* studies have suggested that SLPI reduces migration, invasion and proliferation and induces apoptosis in human HNSCC cells (178,179). In contrast, other studies found that knockdown of *SLPI* decreased the migration, invasion and proliferation of oral carcinoma cells (159,183). *MMP2* and *MMP9* mRNA expression are downregulated in *SLPI*-knockdown oral carcinoma cells, suggesting that SLPI promotes invasion by inducing MMPs (159).

The antiviral activity of SLPI may also be relevant in HNSCC. SLPI can prevent human papillomavirus 16 (HPV16) infection by blocking the epithelial receptor annexin A2, which suggests SLPI could prevent HPV-induced HNSCC (161). Indeed, SLPI is expressed at lower levels in HPV-positive HNSCC than HPV-negative HNSCC (160,184). In addition, SLPI may prevent Epstein-Barr virus (EBV) infection, as SLPI expression is associated with an absence of EBV in nasopharyngeal carcinoma (162). However, the precise role of the antiviral activity of SLPI in the development of HNSCC has not been defined.

Finally, SLPI can inhibit NF-κB signaling in oral premalignant cells *in vitro* (157,179). Activation of NF-κB signaling is associated with the progression from oral premalignant cells to oral squamous cell cancer (157,179); however, whether SLPI actually prevents the progression to HNSCC is unknown.

In conclusion, SLPI is downregulated in HNSCC and may exert both effects protective against cancer—such as antiviral activity and inhibition of NF-κB—as well as cancer-promoting effects, such as stimulating cell invasion.

3.4 SLPI in lung cancer

SLPI is expressed in adenocarcinoma and squamous cell carcinoma of the lung (non-small cell lung cancer, NSCLC) at higher levels than in small cell lung cancer (185). Patients with NSCLC also have higher serum SLPI levels compared to healthy controls (185). Moreover, patients with stage III or IV NSCLC have higher serum SLPI levels than patients with stage I or II NSCLC and serum SLPI decreases after treatment (185), suggesting a relationship between serum SLPI and tumor size or progression. However, the precise function of SLPI in lung carcinoma is unclear.

SLPI stimulates the proliferation of human lung adenocarcinoma cell lines *in vitro* (186). However, other researchers reported SLPI did not affect proliferation or TNF-α-mediated apoptosis in murine lung carcinoma cells (155,187). Transfection of human *SLPI* enhanced subcutaneous tumor growth and the lung-colonizing potential of murine lung carcinoma cells that were intravenously injected into immunocompromised mice (155). Mutant SLPI with lower protease inhibitory capacity did not increase tumor growth and lung-colonizing potential in the same model, suggesting the cancer-promoting mechanism of SLPI is dependent on its protease inhibitory function (155). In contrast, overexpression of SLPI in a liver-metastatic subclone of the same cell line resulted in fewer liver metastases after intrasplenic injection (163). SLPI blocked the induction of *Tnf* mRNA expression in the liver in response to tumor cell infiltration, and this effect was thought to be tumor-promoting in this model (163).

In conclusion, the evidence indicates SLPI is upregulated in NSCLC and may act as a tumor-promoting factor in lung cancer.

3.5 SLPI in gastric cancer

SLPI is overexpressed and associated with shorter five-year survival in gastric carcinoma (188,189). *In vitro*, SLPI promotes the migration, invasion and proliferation of human gastric cancer cells (165,188,189). SLPI upregulates MMP-2 and MMP-9 protein expression via phosphorylation of Elk-1 in gastric cancer cells, which could explain the enhanced invasive ability of SLPI-expressing gastric cancer cells (165). Whether SLPI promotes metastasis in gastric cancer remains to be investigated.

3.6 SLPI in colorectal cancer

The role of SLPI in colorectal cancer is largely unknown. A highly metastatic human colorectal cancer cell line was found to secret increased levels of SLPI compared to the poorly metastatic parental line (164). Additionally, SLPI is expressed in a subgroup of human colorectal carcinomas (190). High SLPI protein expression in colorectal cancer liver metastases was associated with shorter overall survival after resection of the liver metastases (191). In addition, mice subcutaneously injected with SLPI-overexpressing murine colon cancer cells developed tumors more rapidly than mice injected with control cells (171). Therefore, SLPI may play a role in colorectal cancer metastases. However, the exact role of SLPI in human colorectal cancer remains to be elucidated.

3.7 SLPI in pancreatic cancer

SLPI protein expression is upregulated in human pancreatic carcinoma compared to peritumoral tissue (192). Moreover, knockdown of *SLPI* reduced proliferation, migration and invasion and increased apoptosis in human pancreatic cancer cells *in vitro* (192,193). However, *in vivo* studies are needed to reveal whether SLPI promotes tumor growth and metastasis in pancreatic cancer.

3.8 SLPI in prostate cancer

SLPI expression is decreased in prostate carcinoma compared to normal prostate tissue and benign prostatic hyperplasia (194). However, *SLPI* mRNA expression is upregulated in prostate cancer metastases compared to primary tumors, and patients with metastatic prostate cancer have higher serum SLPI levels than patients with localized disease (167). In addition, knockdown of *SLPI* reduced castration-resistant prostate cancer cell proliferation and invasion and increased apoptosis *in vitro* (167). Moreover, overexpression of *SLPI* in human prostate cancer cells led to formation of larger tumors after subcutaneous injection in immunocompromised mice; this tumor-promoting effect was suggested to be due to the ability of SLPI to protect progranulin and confer resistance to TNF-α-induced apoptosis (167). In conclusion, SLPI is low in primary prostate cancer but upregulated in metastases, and SLPI may promote prostate cancer growth and metastasis.

3.9 SLPI in other types of cancer

SLPI protein expression is decreased in cervical adenocarcinoma compared to normal endocervical glands, and was not associated with patient survival (195). In vulvar squamous cell cancer *SLPI* mRNA expression is associated with HPV-negative carcinomas (196). SLPI has been shown to promote the proliferation of human endometrial adenocarcinoma cells, possibly by inducing Cyclin D1 (169). Moreover,

patients with papillary thyroid cancer have higher serum SLPI levels than healthy controls or patients with multinodular nontoxic goiter (197).

3.10 Conclusions

SLPI is upregulated in several types of carcinoma. In addition, SLPI is expressed at higher levels in many metastatic cell lines compared to their poorly metastatic counterparts, suggesting SLPI plays a role in metastasis (163-165,198,199). While the function of SLPI in cancer is yet not entirely clear, most studies indicate SLPI promotes metastasis (see table 2 and figure 2). Some of the *in vitro* and *in vivo* findings on the effect of SLPI on cell proliferation, migration and invasion conflict, possibly due to the complex interactions between SLPI and the factors produced by cells in the tumor microenvironment *in vivo*. SLPI is likely to exert multiple functions at different stages of tumor development and progression, which may not all be cancer-promoting.

In addition, the *in vivo* effects of SLPI in cancer have mostly been studied by injecting human cancer cells into immunocompromised mice (38,155,167,168). Therefore, the possible role of SLPI in anti-cancer immune responses is an important knowledge gap. In view of its strong anti-inflammatory functions in healthy mucosa, SLPI is likely to suppress anti-tumor immune responses as well. We suggest this hypothesis should be tested by examining the associations between SLPI expression in carcinomas and the presence and activity of tumor-infiltrating immune cells.

Surprisingly, the functions of SLPI in tissue damage and chronic inflammation—such as immune regulation, tissue healing and antimicrobial activity—have not yet been studied in cancer. Tumor SLPI expression varies between patients, and high tumor SLPI expression has been linked to a poor prognosis in breast cancer and gastric cancer (38,164,188,189). Therefore, additional study of the role of SLPI in tissue damage and chronic inflammation in different types of cancer is likely to reveal the mechanisms by which SLPI promotes cancer. Finally, the possible link between increased SLPI expression during chronic inflammation and increased SLPI expression in cancer remains to be explored.

Table 2: Function of SLPI in various carcinomas based on human and animal studies

	Prevention of tumor growth or metastasis	Promotion of tumor growth or metastasis
Breast cancer	• Induces apoptosis (*in vitro*) (158,171) • Inhibits tumor growth (*in vivo*) (171)	• Promotes metastasis (*in vivo*) (38,164,170)
Ovarian cancer	• Inhibits cell growth and invasion, and promotes apoptosis (*in vitro*) (177)	• Promotes proliferation and prevents apoptosis (*in vitro*) (166,168) • Promotes tumor growth and metastasis (*in vivo*) (156,168)
HNSCC	• Inhibits cell migration, invasion and proliferation; induces apoptosis (*in vitro*) (178,179)	• Promotes migration, invasion and proliferation (*in vitro*) (159,183)
Lung cancer	• Prevents liver metastasis (*in vivo*) (163)	• Stimulates proliferation (*in vitro*) (186) • Promotes tumor growth (*in vivo*) (155)
Gastric cancer	• Unknown	• Promotes migration, invasion and proliferation (*in vitro*) (165,188,189)
Colorectal cancer	• Unknown	• Promotes tumor development (*in vivo*) (171)
Pancreatic cancer	• Unknown	• Promotes proliferation, migration and invasion; prevents apoptosis (*in vitro*) (192,193)
Prostate cancer	• Unknown	• Promotes proliferation, invasion and prevents apoptosis (*in vitro*) (167) • Promotes tumor growth (*in vivo*) (167)
Endometrial cancer	• Unknown	• Promotes proliferation (*in vitro*) (169)

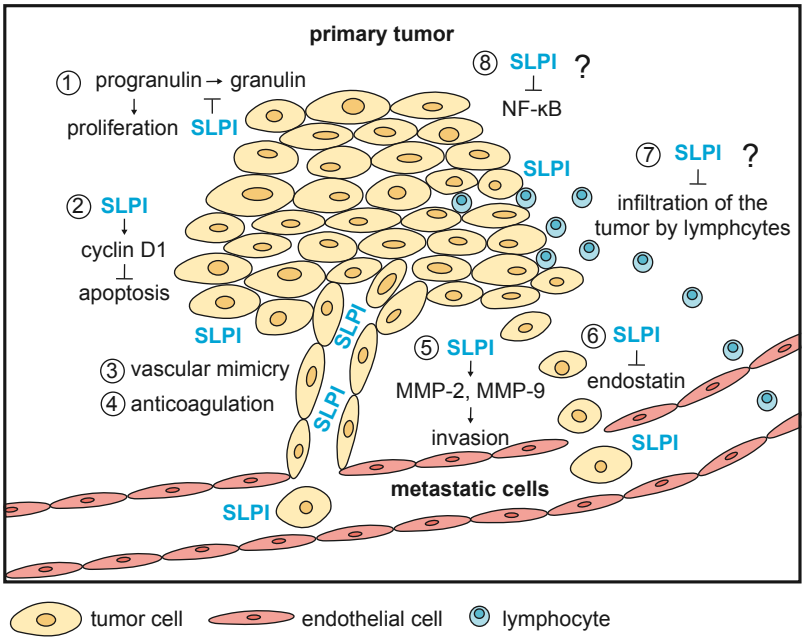

Figure 2: Mechanisms by which SLPI may promote cancer

Production of SLPI by epithelial tumor cells may promote cancer via several mechanisms. (1) SLPI prevents conversion of the epithelial growth factor progranulin to granulin. (2) SLPI promotes cell proliferation by inducing cyclin D1. (3) SLPI induces the formation of vessel-like structures (vascular mimicry), which provide a blood supply to hypoxic regions of the tumor. (4) SLPI acts as an anticoagulant. (5) SLPI promotes tumor cell invasion by inducing MMP-2 and MMP-9 production by tumor cells. (6) SLPI inhibits the antiangiogenic factor Endostatin. (7) We hypothesize that SLPI prevents infiltration of lymphocytes into the tumor. (8) Whether SLPI inhibits activation of NF-κB in tumor cells is unclear.

FUNDING

Sandrine Nugteren was funded by the Dutch Digestive Foundation (grant registration number: Focus Project 15-17).

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CHAPTER 3

High colonic epithelial Secretory Leukocyte Protease Inhibitor (SLPI) expression identifies inflammatory bowel disease patients with extensive neutrophil infiltration and interleukin 17A production

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ABSTRACT

Inflammatory Bowel disease (IBD) comprising Crohn's disease (CD) and ulcerative colitis (UC) is driven by aberrant host-microbial immune interactions. Heterogeneity in disease severity and response to therapy hampers current disease management. Novel treatment strategies tailored to target the patient's underlying immune disease are required. As histology of intestinal biopsies is a key step in the diagnosis of CD and UC we set out to find histological features of intestinal immune processes that identify disease-associated immune responses. Previously, we have identified Secretory Leukocyte Protease Inhibitor (SLPI) as a microbiota-induced regulator of intestinal epithelial responsiveness to microbial signals. We hypothesized that SLPI expression is increased in the inflamed intestine of IBD patients, reflecting local inflammatory responses, and differentiates a subgroup of patients with a distinctive immune profile. underlying immune disease. Here, we show that epithelial SLPI expression is increased in the colon of mice deficient in mucin-2 and in the colon of wild type mice during DSS-colitis, demonstrating that increased epithelial SLPI expression reflects increased microbial contact. Using two independent therapy-naive pediatric IBD cohorts, we show that high colonic epithelial SLPI expression identifies IBD patients with high clinical disease activity, more severe endoscopic and microscopic disease and high numbers of infiltrating neutrophils in the tissue. In addition, RNA sequencing revealed strong activation of neutrophils and distinctive IL-17 signaling in the colon of IBD patients with high epithelial SLPI expression. Moreover, these patients had a Th17 immune protein profile in the peripheral blood. Crucially, gene expression in colonic biopsies from IBD patients with high epithelial SLPI expression was enriched for pathways associated with therapy resistance. In conclusion, our results demonstrate that high colonic SLPI expression at diagnosis identifies IBD patients with severe disease and immune features of therapy resistance, who have increased IL-17A responses and subsequent neutrophilic inflammation compared to patients with low SLPI expression. As such, epithelial SLPI expression is a histological parameter that could support tailored treatment strategies in pediatric IBD patients at time of diagnosis.

INTRODUCTION

Inflammatory bowel disease (IBD) is driven by aberrant host immune responses to harmless microbial antigens, leading to chronic gastrointestinal inflammation. Based on clinical and pathological characteristics, patients are classified into one of the two major clinical forms of IBD, Crohn's disease (CD) or ulcerative colitis (UC) with overlapping treatment strategies. However, even within these clinical subtypes, these diseases are heterogeneous in regard to disease location, disease behavior, treatment response and course of disease. Upon insufficient control of inflammation, patients are at risk of developing complications such as strictures (in CD) and the need for surgery (both in CD and UC). In pediatric IBD, anti-tumor necrosis factor alpha (TNF-α) biologicals are successful in inducing remission in the majority of patients with moderate to severe relapsing pediatric IBD, but up to 40% of patients lose response to anti-TNF-α treatment in the long term (1-4). Although IBD is an immune-mediated disease, there are no robust immune parameters to classify the heterogenous intestinal disease in relation to clinical disease course and treatment response (5). New developments in systems analysis allow comprehensive analysis of complex immune responses but require simplification for applicability in clinical practice (6,7). Histological evaluation of intestinal biopsies is a key step in the diagnosis of IBD. Identifying histological parameters that differentiate intestinal immune processes at time of diagnosis could provide a step towards patient immune classification.

Recent evidence argues for a role of neutrophils in determining a complicated IBD disease course and therapy resistance. Persistent histological disease activity, in particular tissue infiltration by neutrophils despite endoscopically normal mucosa, predicts relapse in UC patients (8,9). Interestingly, high expression of Oncostatin M (OSM), a neutrophil product, in the inflamed intestine of IBD patients is associated with severe histological disease and nonresponse to anti-TNF-α treatment (10,11). However, neutrophils are a heterogeneous population with highly varying immune activity and the reason why neutrophil recruitment and activation are associated with poor disease outcome in IBD is unclear (12). Microbial-epithelial interaction and subsequent cytokine and chemokine production by epithelial cells and fibroblasts orchestrate neutrophil infiltration and function. Therefore, investigating epithelial function in intestinal biopsies at time of diagnosis could help dissect the epithelial-neutrophil-inflammation axis in individual IBD patients.

We have previously found that Secretory Leukocyte Protease Inhibitor (SLPI) regulates intestinal epithelial chemokine production in response to microbiota (13). SLPI is expressed mainly by epithelial cells, including the epithelium lining the

gastrointestinal and respiratory tract (14,15). SLPI prevents tissue damage by inhibiting proteases including neutrophil elastase (16). In addition, SLPI inhibits nuclear factor kappa B (NF-κB) mediated inflammatory gene transcription, thereby regulating the threshold of innate immune responses to microbial signals (17-19). Moreover, SLPI has broad antibacterial, antifungal and antiviral properties (20-23). SLPI expression in the intestinal epithelium is induced upon repetitive microbial contact, as seen during colonization of germ-free mice (13). Therefore, we hypothesized that high intestinal epithelial SLPI expression reflects increased contact between epithelial cells and micro-organisms. As SLPI neutralizes neutrophil elastase and suppresses production of the neutrophil attractant C-X-C motif chemokine ligand 8 (CXCL8) (13), we anticipated that epithelial SLPI expression is especially increased in tissues heavily infiltrated by inflammatory neutrophils. Therefore, we questioned whether high epithelial SLPI expression in intestinal biopsies identifies a subgroup of IBD patients with a distinctive immune response and distinct clinical disease subtype.

Here, we demonstrate that immunohistochemical detection of a single epithelial protein SLPI in colonic biopsies can be used to classify IBD patients at time of diagnosis irrespective of whether they have colonic CD or UC. Patients with high intestinal SLPI protein expression have increased endoscopic severity of inflammation and increased histological disease- and clinical disease- activity. On histology, high SLPI expression co-localizes with abundant neutrophil infiltration. Paired biopsy mRNA analyses of SLPI^{high} biopsies demonstrate a signature of activated neutrophilic inflammation including higher expression of the genes *OSM, IL-1B*, *and CXCL8,* which have been associated with severe disease progression and resistance to anti-TNF treatment. This high SLPI-inflammatory neutrophil network may be amplified by T-cell derived IL-17A, which can further stimulate SLPI expression in epithelial cells *in vitro* and is more abundant in intestinal lesions and the circulation of SLPI^{high} patients.

MATERIALS AND METHODS

PIBD-SETQuality subcohort

As part of the PIBD Network for Safety, Efficacy, Treatment and Quality improvement of care (PIBD-SETQuality), patients < 18 years old suspected of IBD were included at time of diagnostic endoscopy (24). Patients were diagnosed according to the revised Porto criteria (25). For this study, biopsies collected at diagnostic colonoscopy from a subcohort of 78 treatment-naïve patients were available. The diagnostic colonoscopy from all patients had been performed at the Erasmus Medical Center in Rotterdam, The Netherlands. All patients and parents signed informed consent for collection of biomaterials. This study was approved by the Medical Ethical Committee of the

Erasmus University Medical Centre-Sophia Children's Hospital Rotterdam (METC number: trial registration number NCT03571373) (24). Endoscopic disease activity was scored according to the Simple Endoscopic Score for Crohn Disease (SES-CD) (26) for CD patients and the Ulcerative Colitis Endoscopic Index of Severity (UCEIS) (27) for UC patients. As the original UCEIS is determined by the score of the most inflamed segment only (range 0 to 8), for our study purpose, we also calculated the sum of the UCEIS of each colonic segment to have a score representing the whole colon (range 0 to 40). The SES-CD and total UCEIS were only analyzed for patients who had complete ileocolonoscopy. Clinical disease activity was scored by using the weighted Pediatric Crohn's Disease Activity Index (PCDAI) (28) for CD patients and the Pediatric Ulcerative Colitis Activity Index (PUCAI) (29) for UC patients and by categorizing these into four groups ('none', 'mild', 'moderate', 'severe') based on validated cut-offs for these scores. Disease location, disease behavior and disease extent were scored according to the Paris classification (30). Further details on this cohort and collection of clinical data have been described previously (24).

Biopsies were collected from ileum and colon (from macroscopically inflamed as well as non-inflamed areas), when possible and applicable. Two adjacent 'paired' biopsies were collected from each location: one for histological analysis and one for RNA sequencing. Biopsies for histological analysis were fixed in 4% paraformaldehyde for 4 hours at room temperature and subsequently stored in 70% ethanol at 4°C for at least 12 hours and at most 3 weeks. Tissues were subsequently embedded in paraffin and 4-micrometer-thick sections were cut and mounted on polysine adhesion glass slides (Thermo Fisher Scientific, Bleiswijk, the Netherlands). Biopsies for RNA sequencing were collected in RNA*later*® tissue storage reagent (Sigma-Aldrich, Zwijndrecht, The Netherlands) overnight at 4°C and subsequently stored at -80°C.

EDTA blood was collected and centrifuged at a relative centrifugal force of 321 for 10 minutes at room temperature. The top layer of plasma was collected and stored at -80°C.

Longitudinal pediatric IBD cohort

In this cohort, therapy-naïve patients < 18 years old suspected of IBD were included at time of diagnostic colonoscopy from 2007 until 2020. Patients were diagnosed according to the revised Porto criteria (25). Biopsies were collected at diagnostic colonoscopy at the Erasmus Medical Center in Rotterdam, The Netherlands. All patients and parents signed informed consent for collection of biomaterials. This study was approved by the Medical Ethical Committee of the Erasmus University Medical Centre-Sophia Children's Hospital Rotterdam (METC 2007-335).

Biopsies were collected from ileum and colon (from macroscopically inflamed as well as non-inflamed areas), when possible and applicable. All biopsies were collected in RNA*later*® tissue storage reagent (Sigma-Aldrich) overnight at 4°C and subsequently stored at -80°C.

Muc2-deficient mice

Mucin 2-deficient (Muc2) mice were generated as described previously (31). Muc2-deficient mice were co-housed with wild type littermates. Distal colonic tissue was collected for histological analysis and was fixed in 4% paraformaldehyde and embedded in paraffin as described previously (31). Five-micrometer-thick sections were cut from paraffin blocks and mounted on polysine adhesion glass slides (Thermo Fisher Scientific). In addition, RNA was extracted from distal colonic tissue and cDNA was synthesized for quantitative PCR as described previously (31).

Dextran sulfate sodium (DSS)-colitis

In total 11 C57/Bl6 wild type mice (both males and females) received 2% dextran sulfate sodium (TdB consultancy AB, Uppsala, Sweden) in the drinking water ad libitum for 5 days in two independent experiments. In addition, 3 C57/Bl6 wild type littermates were left untreated. All mice were reared under specific pathogen free conditions. Feces samples were collected daily on day 1 until day 10 and were kept on ice and subsequently stored at -80°C. Mice were sacrificed on day 5, day 10 or day 36 and distal colonic tissue was fixed in 4% paraformaldehyde for 4 hours at room temperature and subsequently stored in 70% ethanol at 4°C for at least 12 hours and at most 3 weeks. Subsequently, the tissue was embedded in paraffin and 4-micrometer-thick sections were cut and mounted on polysine adhesion glass slides (Thermo Fisher Scientific). In addition, adjacent distal colonic tissue was collected in RNA*later*® tissue storage reagent (Sigma-Aldrich) overnight at 4°C and subsequently stored at -80°C. This animal experiment was approved by and performed in accordance with the animal experimental committee of the Erasmus Medical Center in Rotterdam, the Netherlands. DSS-induced intestinal damage was scored according to a scoring system for inflammation-associated histological changes during DSS colitis as described by Wirtz et al. (32).

Culture of TR146 cells

The buccal epithelial cell line TR146 (previously described (13)) was cultured in Dulbecco's Modified Eagle's Medium with glutamax (DMEM, Gibco™, Thermo Fisher Scientific; #31966-021) supplemented with 10% heat-inactivated fetal calf serum (FCS, Bodinco BV, Alkmaar, the Netherlands; #BDC-1098), 1x non-essential amino acid solution (Gibco™, Thermo Fisher Scientific; #11140-035) and 25 U/mL

penicillin-streptomycin (Gibco™, Thermo Fisher Scientific; #15070-063) according to manufacturer's instructions. Cells were plated on 24-wells culture plates (Greiner Bio-One International GmbH, Alphen aan den Rijn, the Netherlands) until they reached 90-100% confluency and were subsequently starved from serum for 4-24 hours using DMEM without glutamax before stimulation. Stimulation was performed with recombinant human interleukin-1 beta (IL-1β, Immunotools GmbH; #11340013), recombinant human TNF-α (BD Biosciences, San Jose, California, USA; #554618), recombinant human interferon gamma (IFN-γ, Immunotools GmbH, Friesoythe, Germany; #11343536) or recombinant human IL-17A (BioLegend, San Diego, CA, USA; #570502) for 16 hours at concentrations as written in figure legends. Cells were lysed and collected in RA1 lysis buffer (Macherey-Nagel GmbH & Co. KG, Dueren, Germany) + 20mM dithiothreitol (DTT, Sigma-Aldrich) and stored at -80°C. In addition, supernatant was collected after 16 hours and stored at -20°C for measurement of SLPI protein as written below.

Measurement of human SLPI protein in supernatant

Human SLPI was measured in the supernatant of TR146 cells by enzyme-linked immunosorbent assay (ELISA). A 96-wells high binding surface flat-bottom plate (Corning®; #9018) was coated with 0.5 µg/mL monoclonal antibody against human SLPI (R&D Systems/Bio-Techne, Minneapolis, MN, USA; MAB1274; clone 20409) in PBS overnight at room temperature. Washing was performed with 0,05% TWEEN 20 (Sigma-Aldrich) in PBS. The plate was blocked with 10% fetal calf serum (Bodinco B.V., Alkmaar, the Netherlands) in PBS for 1 hour at room temperature. Samples were incubated for 2 hours at room temperature. Recombinant human SLPI (R&D Systems/Bio-Techne; 1274-PI-100) was used to generate a standard curve (31.25 – 2000 pg/mL). To detect SLPI, the plate was incubated with 0.1 µg/mL biotinylated polyclonal antibody against human SLPI (R&D Systems/Bio-Techne; BAF1274) for 1 hour at room temperature and subsequently incubated with 1:5000 horseradish peroxidase-conjugated streptavidin (BD Biosciences; #554066) in the dark at room temperature for approximately 1 hour. 3, 3',5 ,5'-Tetramethylbenzidine Liquid Substrate (Sigma-Aldrich; T4444) was used for detection of the horseradish peroxidase. The reaction was stopped using 1M H_3PO_4. Signal was detected at 450+570 nanometer on a VersaMax reader (Molecular Devices, San Jose, CA, USA).

Measurement of SLPI in murine feces

Murine SLPI was measured in murine feces by ELISA. A 96-wells high binding surface flat-bottom plate (Corning®; #9018) was coated with 0.2 µg/mL polyclonal antibody against mouse SLPI (R&D Systems/Bio-Techne; AF1735) in bicarbonate buffer (pH 9.5) overnight at 4°C. Washing was performed with 0.05% TWEEN 20 (Sigma-Aldrich) in PBS.

The plate was blocked with 10% fetal calf serum (Bodinco B.V.) in PBS for 1 hour at room temperature. Samples were incubated for 2 hours at room temperature. Recombinant mouse SLPI (MyBioSource, Inc., San Diego, CA, USA; BMS2012482) was used to generate a standard curve (up to 250 ng/µL). To detect SLPI, the plate was incubated with 0.4 µg/mL biotinylated polyclonal antibody against mouse SLPI (R&D Systems/Bio-Techne; BAF1735) for 1 hour at room temperature and subsequently incubated with 1:5000 horseradish peroxidase-conjugated streptavidin (BD Biosciences; #554066) in the dark at room temperature for approximately 1 hour. 3, 3',5 ,5'-Tetramethylbenzidine Liquid Substrate (Sigma-Aldrich; T4444) was used for detection of the horseradish peroxidase. The reaction was stopped using 1M H_3PO_4. Signal was detected at 450+570 nanometer on a VersaMax reader (Molecular Devices). Total protein was measured in murine feces by performing a bicinchoninic acid assay (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific) according to manufacturer's instructions.

Hematoxylin & eosin staining

Sections were deparaffinized in xylene and rehydrated in ethanol. Subsequently, sections were stained with hematoxylin (Vector Laboratories, Vector Burlingame, CA, USA) for 7 minutes and subsequently stained with eosin (Eosin Yellowish, Sigma-Aldrich) for 7 minutes. Finally, sections were dehydrated and immersed in xylene and mounted in Entallan™ (Sigma-Aldrich).

Immunohistochemistry

Sections were deparaffinized in xylene and rehydrated in ethanol. Subsequently, sections were incubated in 3% H_2O_2 in PBS for 20 minutes to quench endogenous peroxidase activity. For human tissue, antigen retrieval was performed by microwave treatment in citrate buffer (10mM, pH 6.0). For murine tissue, antigen retrieval was performed by treating sections with 0.1% pepsin from porcine gastric mucosa (Sigma-Aldrich; P7000) in 0.01N HCl for 7 minutes at 37°C. Subsequently, sections were blocked for 1 hour at room temperature in Tris buffer (10mM, pH 8.0) containing 5mM EDTA (pH 8.0), 0.15M NaCl, 0.25% gelatin, 0.05% Tween-20 and 10% normal human serum (AB serum, Sanquin, Amsterdam, The Netherlands) for human tissue or 10% normal mouse serum (Thermo Fisher Scientific) for murine tissue, plus 10% of the serum matching the species in which the secondary antibody was raised (see table below). Subsequently, sections were stained with the primary antibody in PBS overnight at 4°C. Immunoreactive sites were detected by incubation with the biotinylated secondary antibody for 1 hour at room temperature. Biotinylated antibodies were detected using a complex of avidin and biotin (Vectastain ABC Elite Kit, Vector Laboratories) and 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich). Sections were

counterstained with hematoxylin (Vector Laboratories) and subsequently dehydrated and immersed in xylene and mounted in Entallan™ (Sigma-Aldrich).

protein	serum	primary antibody	secondary antibody
human SLPI*	normal horse serum (Biowest, Nuaillé, France)	anti-human SLPI monoclonal mouse IgG1 antibody, 4 µg/mL (HycultBiotech, Uden, The Netherlands; HM2037, clone 31)	horse-anti-mouse antibody, 1:500 (Vector Laboratories)
human SLPI	normal rabbit serum (Jackson ImmunoResearch, West Grove, PA, USA)	anti-human SLPI polyclonal goat IgG antibody, 1µg/mL (R&D Systems/Bio-Techne; BAF1274)	rabbit-anti-goat antibody, 1:500 (Vector Laboratories)
human S100A8/A9	normal horse serum (Biowest)	anti-human S100A8/S100A9 heterodimer monoclonal mouse IgG1 antibody, 1.2 µg/mL (R&D Systems/Bio-Techne; MAB45702)	horse-anti-mouse antibody, 1:500 (Vector Laboratories)
human IL-17	normal rabbit serum (Jackson ImmunoResearch)	anti-human IL-17/IL-17A polyclonal goat IgG antibody, 2 µg/mL (R&D Systems/Bio-Techne; AF-317)	rabbit-anti-goat antibody, 1:500 (Vector Laboratories)
murine SLPI	normal rabbit serum (Jackson ImmunoResearch)	anti-mouse SLPI polyclonal goat IgG antibody, 2 µg/mL (R&D Systems/Bio-Techne; AF1735)	rabbit-anti-goat antibody, 1:500 (Vector Laboratories)

*The monoclonal antibody was used to detect SLPI, unless otherwise indicated.

Image acquisition

Images were acquired using a Leica DM5500B microscope equipped with a Leica DFC450 C camera and analyzed in Leica Application Suite X software (Leica Microsystems, Rijswijk, the Netherlands).

Scoring of human epithelial SLPI protein intensity

The intensity of SLPI protein expression in the cytoplasm of epithelial cells was scored in a semi-quantitative manner as 'negative', 'weak', or 'strong'. Per biopsy, the strongest intensity was scored. The scoring strategy was designed based on the range of SLPI staining intensity observed in all intestinal biopsies and was designed separately for SLPI detected with the monoclonal antibody and the polyclonal antibody. The observer was blinded to the clinical information at time of assessment.

Scoring of global histological disease activity

H&E-stained biopsies from the PIBD-SETQuality subcohort were scored by an experienced gastro-intestinal pathologist using a modification of the Global Histological disease Activity Score (GHAS) (33). In the modified GHAS, the parameter 'presence of granulomas' was excluded because the cohort included both biopsies from CD and UC-patients, and the parameter 'number of biopsies affected' was excluded because in this study at most two biopsies were taken from the same anatomic region, according to a modification of the GHAS as described by Li et al. (34). In addition, for the 'activity GHAS' subscore, the parameters 'architectural changes' and 'infiltration of mononuclear cells in the lamina propria' were excluded to be able to study disease activity as opposed to chronic changes (34).

For selection of the most affected ileal and colonic biopsy per patient, the biopsy with the highest modified GHAS as scored by one of the authors was chosen. In case of multiple biopsies with the same modified GHAS, a biopsy taken from a macroscopically inflamed region was chosen over a biopsy taken from a macroscopically non-inflamed region. Observers were blinded to the clinical information at time of assessment.

Olink® Proximity Extension Assay (PEA)

Multiplex PEA using the Olink Target 96 Inflammation panel (#95302) was performed on patient plasma by Olink (Olink Proteomics, Uppsala, Sweden) (35). In short, an antibody-based immunoassay merged with qPCR detection results in quantification of multiple proteins simultaneously. Data are expressed as normalized protein expression (NPX), an arbitrary unit on log2 scale acquired by normalization of qPCR values reflecting relative protein abundance.

Quantitative PCR (qPCR)

Total RNA was extracted from tissue using the NucleoSpin RNA extraction kit for isolation of RNA (Macherey-Nagel GmbH & Co) according to manufacturer's instructions. Quantity and purity of extracted RNA was assessed using a DeNovix DS-11 spectrophotometer (DeNovix Inc., Wilmington, DE, USA). A maximum of 1000 nanogram mRNA was used to synthesize cDNA using the SensiFAST cDNA Synthesis Kit (Bioline, London, United Kingdom). Real-time qPCR was performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Veenendaal, The Netherlands) using the SensiMix™ SYBR® Hi-ROX Kit (Bioline). Relative expression was calculated as $2^{-(\text{Ct value housekeeping gene} - \text{Ct value gene of interest})}$, using housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for human targets and cyclophilin A for murine targets.

gene	forward primer	reverse primer
GAPDH (human)	GTCGGAGTCAACGGATT	AAGCTTCCCGTTCTCAG
SLPI (human)	TCCAGGGAAGAAGAGATGT	TGCCCATGCAACACTT
S100A8 (human)	GTTGACCGAGCTGGAGA	GCACCCTTTTTCCTGATATAC
S100A9 (human)	TCCTCGGCTTTGACAGA	CGCACCAGCTCTTTGA
Cyclophilin A (murine)	AACCCCACCGTGTTCT	CATTATGGCGTGTAAAGTCA
SLPI (murine)	GTGACGGCAAATACAAGTG	GAGCCAAAAGGAGATGTTAGT

RNA sequencing

Per patient, one ileal and one colonic biopsy were selected based on the highest modified GHAS in the paired biopsy, as described above. If the RNA quality from that biopsy was insufficient (see below), the biopsy from the region with the second highest modified GHAS in the paired biopsy was used.

Capillary gel electrophoresis was applied to assess the RNA quality. Briefly, RNA was inserted in an Agilent 6000 Nano Chip (25-250 ng/µL; when >250 ng/µL samples were diluted) and loaded into the BioAnalyzer (Agilent, Santa Clara, CA, United States) according to manufacturer's instructions. An appropriate eukaryotic total RNA assay was chosen and the 2100 Expert software (Agilent) was used to calculate the RNA integrity number (RIN). If the RNA concentration was below 25 ng/µL or if the RIN could not be calculated, RNA was inserted in an Agilent 6000 Pico Chip (250-5000 pg/µL; when >5000 pg/µL samples were diluted) and the RIN was calculated again. Samples with a RIN < 6 were excluded.

Library preparation was performed on 500 ng total RNA, using the KAPA mRNA HyperPrep Kit (Roche, F. Hoffmann-La Roche AG, Basel, Switzerland) according to manufacturer's instructions. Briefly, mRNA was captured via hybridization to oligo(dT)-conjugated magnetic beads. After washing and elution, mRNA was fragmented at high temperature in a magnesium-containing solution, and primed with random primers. mRNA was then reverse transcribed, followed by a second strand synthesis. The dsDNA molecules were tailed with deoxyadenosine at the 3' ends, and adapters were then ligated to the overhangs. DNA was precipitated on magnetic beads and washed with ethanol. Elution of the DNA allowed further amplification via PCR with primers that anneal to the adapters, flanked by Illumina P5 and P7 sequences (Illumina, Inc., San Diego, California, U.S.). The concentration and size distribution of these libraries were measured using the Qubit ds HS Assay (Thermo Fisher Scientific) and High Sensitivity DNA Kit for BioAnalyzer (Agilent), respectively. Libraries were diluted to 2nM and paired-end sequenced on a Novaseq 6000 (Illumina, Inc.) with 2 x 100 read-length at an expected library size of approximately 40 million reads per sample.

Statistical analyses & visualization

For analysis of the RNA sequencing data and Olink PEA data the PIBD-SETQuality subcohort was dichotomized in a SLPI^{low} and SLPI^{high} group based on SLPI immunohistochemistry score for the paired biopsy. The cut-off was chosen between negative (SLPI^{low} group) and weak/strong (SLPI^{high} group), as this resulted in the most equal distribution of patients over the two groups.

Statistical analyses and visualization were performed using R version 3.5.1 (36) for all analyses except for analysis of the Olink PEA data (see below). The R package ggplot2 (37) was used for visualization. The R packages vipor (38) and ggbeeswarm (39) were used to generate violin plots and the R package pheatmap (40) was used to generate heatmaps. In each violin plot, all violins have the same area before tails are trimmed. The statistical tests used are indicated in the figure legends. Read counts and Transcripts Per Kilobase Million (TPM)-values per transcript isoform were determined with Salmon version 1.4.0 (41) using Ensembl v104 for GRCh38 as the target transcriptome and using the remainder of the genome as decoy. Gene counts were summarized from the Salmon transcript isoform estimates using the R packages tximport version 1.22 (42). The summarized gene counts were in turn normalized, prefiltered by removing genes with consistent low expression across all samples or with extreme outliers among one or two samples, and subsequently used for differential gene expression analysis between groups of interest using DESeq2 v1.34.0 (43) at default parameter values. DESeq2 *P*-values (calculated using the Wald test) were corrected for multiple testing using the Benjamini and Hochberg procedure (44). Differentially expressed genes of interest had a false discovery rate (FDR)-adjusted *P*-value ≤ 0.05 and log2 fold change > 1 or < -1. GSEA was performed with fgsea v1.20 (45) using predefined gene sets from the Molecular Signatures Database (MSigDB v7.5.1). Gene lists were ranked on the basis of the log2 fold change or the ashr method (46) made available through the DESeq2 package. Classical enrichment statistics with 1.000.000 permutations was used to determine significant enrichment within gene sets.

Analysis of the Olink PEA data was performed using Python. Identification of differentially expressed proteins in plasma was compared between the SLPI^{low} and SLPI^{high} group using independent T-tests with Benjamini-Hochberg correction Normalized Protein Expression (NPX)-values. Samples were clustered on differentially expressed proteins using hierarchical clustering with Ward's method using the Ward2 algorithm (47,48). Clustering was applied on healthy control-adjusted Z-scores by first subtracting the average NPX value of 43 healthy control and IBD-negative patients and subsequently dividing by the standard deviation.

RESULTS

SLPI expression is increased in the colonic epithelium upon microbial translocation in two murine experimental colitis models

Epithelial SLPI expression is driven by microbial interaction (13,49,50). Therefore, we questioned whether increased contact between epithelial cells and micro-organisms, as occurs in IBD, increases epithelial SLPI expression. To answer this question, we assessed colonic SLPI expression in mice deficient in mucin 2 (Muc2). Muc2 is the main secretory mucin expressed by the human and murine colonic epithelium and is therefore an important component of the intestinal mucus layer (51,52). In mice deficient in Muc2, bacteria are in close contact with the epithelium and translocate into the crypts (53). In consequence, mice deficient in Muc2 develop spontaneous colitis (31). We observed significantly increased *Slpi* mRNA expression in the distal colon of Muc2 deficient mice compared to wild type littermates at 4 weeks and 8 weeks after birth (Figure 1a). In addition, epithelial cells in the distal colon of Muc2 deficient mice showed more intense SLPI protein expression compared to wild type littermates at 2 weeks, 4 weeks and 8 weeks after birth (Figure 1b). SLPI expression was most abundant in large cells scattered throughout the epithelium (Figure 1b), which is in agreement with previous reports demonstrating SLPI production in intestinal goblet-type epithelial cells (15).

To further assess the relationship between SLPI and microbiota-induced colitis, we assessed changes in epithelial SLPI expression during dextran sulfate sodium (DSS)-colitis. DSS-colitis results from a compromised barrier integrity leading to exposure to luminal antigens (32). We induced DSS-colitis in wild type mice, resulting in damage and inflammation in the distal colon with a peak at day 10 after start of DSS treatment (Figure 1c). *Slpi* mRNA expression was significantly increased in the distal colon of wild type mice at day 10 compared to untreated mice and returned to baseline levels at day 36 (Figure 1d). In addition, we detected an increase in excretion of SLPI protein in feces, with a peak at day 8 (Figure 1e). Microscopically, we observed many SLPI-positive epithelial cells in the distal colon at day 10 and day 36, whereas we found few SLPI-positive cells in the distal colon at day 5 and in untreated mice (Figure 1f). SLPI-positive epithelial cells were mainly large goblet-type cells. In conclusion, colonic SLPI expression is increased in both the tissue and feces at the peak of colitis induced by bacterial translocation. These findings argue that increased colonic epithelial SLPI expression is a response to increased microbial contact and subsequent microbial translocation.

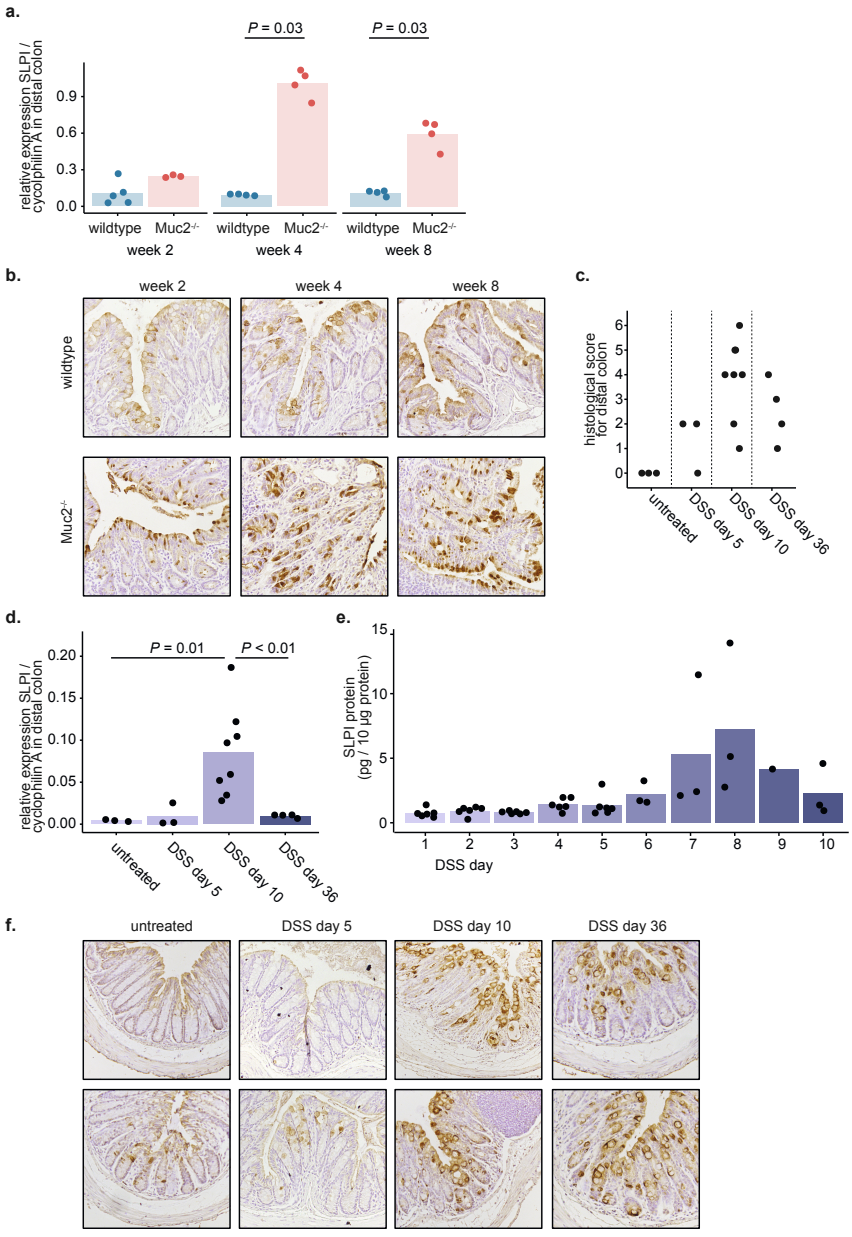

Figure 1: SLPI expression is increased in the distal colon of Muc2-/- mice and in the distal colon of wild type mice during DSS-colitis

Slpi mRNA expression was measured in distal colonic tissue of Muc2 deficient (Muc2$^{-/-}$) mice and wild type littermates at 2 weeks, 4 weeks, and 8 weeks after birth (a). Bars represent the mean relative expression of multiple mice and *P*-values were calculated using the Wilcoxon rank sum test (a + d). SLPI protein expression was detected by immunohistochemistry in distal colonic tissue of Muc2$^{-/-}$ mice and wild type littermates at 2 weeks, 4 weeks, and 8 weeks after birth (b). Representative images were acquired at 20x magnification (b). Wild type mice received 2% DSS in the drinking water for 5 subsequent days in two independent experiments (c-f). Histological damage and inflammation were scored on a scale from 0 to 6 on H&E-stained sections of the distal colon (c). *Slpi* mRNA expression was measured in the distal colon of untreated mice and at day 5, day 10 and day 36 of DSS treatment (d). SLPI protein excretion was measured in feces on day 1 until day 10 of DSS treatment (e). SLPI protein levels are shown relative to total protein levels for mice from which feces was available on that day (e). Bars represent the mean relative SLPI protein levels (e). SLPI protein expression was detected by immunohistochemistry in the distal colon of untreated mice and at day 5, day 10 and day 36 of DSS treatment (f). Representative images of the distal colon of two mice per condition were acquired at 10x magnification (f).

SLPI expression is increased in the colonic epithelium of therapy-naive pediatric IBD patients

We next questioned whether intestinal epithelial SLPI expression is increased during human intestinal inflammation. Therefore, we searched in the public genomic repository Gene Expression Omnibus (GEO) for SLPI mRNA expression in microarray data from intestinal biopsies of two adult IBD patient cohorts. In the Arijs 2009 cohort, mRNA was isolated from ileal and colonic biopsies taken from controls (healthy adults) and from macroscopically inflamed mucosa of adult IBD patients refractory to corticosteroids and/or immunosuppression (54). *SLPI* mRNA expression was significantly higher in colonic biopsies compared to ileal biopsies (Wilcoxon rank sum test: $P < 0.01$, Figure 2a). In addition, *SLPI* RNA expression was significantly increased in ileal biopsies from CD patients compared to controls (Figure 2a). In colonic biopsies, *SLPI* mRNA expression was higher in CD and UC patients compared to controls, but these differences were not statistically significant (Figure 2a). In the Arijs 2017 cohort, microarrays were used to analyze mRNA expression in ileal and colonic biopsies from controls (healthy adults) and adult IBD patients with either active disease or inactive disease as endoscopically assessed (55). *SLPI* mRNA expression was also higher in colonic biopsies compared to ileal biopsies in this cohort (Wilcoxon rank sum test: $P < 0.01$, Figure 2b). In addition, *SLPI* mRNA expression was higher in ileal biopsies from CD patients with active disease compared to controls and compared to CD patients with inactive disease (Figure 2b). In the colon, *SLPI* mRNA expression was higher in both biopsies from CD patients with active disease and in biopsies from UC patients with active disease compared to controls (Figure 2b). *SLPI* mRNA expression was not different between colonic biopsies from UC patients with active and inactive disease (Wilcoxon rank sum test: $P = 0.73$, Figure 2b). Taken together, these data show that *SLPI* mRNA expression is increased in the intestine of adult IBD patients and may be related to macroscopic disease activity.

Both the IBD patients in the Arijs 2009 cohort and in the Arijs 2017 cohort were not therapy-naive. Therefore, to further corroborate these data we next assessed *SLPI* mRNA expression in ileal and colonic biopsies from therapy-naive pediatric CD patients, UC patients and IBD-negative patients in our Longitudinal pediatric IBD cohort. Overall, in health and disease, *SLPI* mRNA expression was higher in colonic biopsies compared to ileal biopsies (Wilcoxon rank sum test, $P < 0.01$). During inflammation SLPI mRNA was increased as illustrated by higher expression in biopsies taken from macroscopically inflamed regions compared to macroscopically non-inflamed regions (Wilcoxon rank sum test; ileum: $P < 0.01$; colon: $P < 0.01$; Figure 2c). In CD patients *SLPI* mRNA expression was higher in both ileal and colonic biopsies compared to IBD-negative patients (Figure 2c). In UC patients *SLPI* mRNA expression in colonic biopsies was higher compared to IBD-negative patients (Figure 2c). In line with the fact that most ileal biopsies from UC patients were macroscopically non-inflamed *SLPI* mRNA expression was not significantly higher in ileal biopsies from UC patients compared to IBD-negative patients (Figure 2c). When comparing SLPI mRNA expression in the inflamed colonic biopsies of CD and UC patients, there was no significant difference (Figure 2c). In conclusion, these data demonstrate that *SLPI* mRNA is increased in both the ileum and colon of untreated pediatric IBD patients compared to IBD-negative patients and that SLPI expression is associated with macroscopic inflammation.

To further explore whether histological detection of SLPI using immunohistochemistry could differentiate intestinal immune processes between IBD patients at time of diagnosis, we analyzed SLPI in the well-characterized PIBD-SETQuality cohort, an inception cohort with biological material for all patients included in Rotterdam (24). First, we performed immunohistochemistry for SLPI on ileal and colonic biopsies from therapy-naive pediatric IBD patients and IBD-negative patients. To robustly assess epithelial SLPI expression, we detected SLPI protein by immunohistochemistry using two different antibodies: a monoclonal antibody raised against human SLPI purified from sputum and a polyclonal antibody raised against *Escherichia coli*-derived recombinant human SLPI. Using either the monoclonal or the polyclonal antibody, we detected SLPI protein expression in the intestine mainly in epithelial cells (Figure 2d + Supplementary figure 1a). Consistent with our observations in mice, SLPI protein expression was most abundant in large goblet-like cells in the colon (Figure 2d + Supplementary figure 1a). To compare SLPI protein expression between IBD patients and IBD-negative patients, we used the maximum SLPI score for macroscopically inflamed and macroscopically non-inflamed ileum and colon (Figure 2e + Supplementary figure 1b). SLPI protein expression was higher in colonic biopsies from macroscopically inflamed regions compared to colonic biopsies from macroscopically non-inflamed regions (Figure 2e + Supplementary figure 1b). Importantly, SLPI protein

expression was higher in colonic biopsies from CD and UC patients compared IBD-negative patients (Figure 2e + Supplementary figure 1b). Detection of SLPI protein with the monoclonal antibody was significantly associated with detection of SLPI with the polyclonal antibody in both ileal and colonic biopsies from IBD patients and IBD-negative patients (two-sided Fisher's exact test; ileum: n = 76, $P < 0.01$; colon: n = 114, $P < 0.01$; Supplementary figure 1c). As the monoclonal antibody showed less background staining (Figure 2d + Supplementary figure 1a), we further focused on the results obtained with the monoclonal antibody.

To test whether colonic epithelial SLPI expression is related to clinical disease activity in IBD, we grouped IBD patients based on the SLPI immunohistochemistry score for the most affected colonic biopsy, as defined by the highest modified Global Histological disease Activity Score (GHAS). Patients with UC had significantly higher SLPI scores compared to patients with CD (Figure 2f + Supplementary figure 2). In two patients with UC SLPI protein was not detected in the selected "most affected colonic biopsy" (Figure 2f); one of these patients had ulcerative proctitis without macroscopic inflammation in the colon. The second patient appeared to have mild disease resulting in a macroscopically non-inflamed colon biopsy being selected for analysis based on the highest GHAS score which was 3 out of 12. Interestingly, IBD patients with high SLPI scores significantly more often had moderate to severe disease activity compared to no or mild disease activity, indicating that high colonic epithelial SLPI expression is associated with more active disease at diagnosis (Figure 2f + Supplementary figure 2). CD patients with high SLPI scores had significantly more severe endoscopic disease as assessed by the SES-CD (Figure 2g + Supplementary figure 2). As expected, CD patients with high colonic SLPI scores relatively more often had colonic or ileocolonic disease compared to ileal disease (Supplementary figure 2). In patients with UC, we observed a trend between high SLPI scores and endoscopic disease as assessed by the UCEIS or by the sum of the UCEIS of each colonic segment (total UCEIS), but these trends were not statistically significant (Figure 2g + Supplementary figure 2).

In conclusion, we show that SLPI protein and mRNA expression are increased in the colonic epithelium of both CD and UC patients compared to non-IBD patients. High colonic epithelial SLPI protein expression, detected by immunohistochemistry, is associated with more severe clinical disease activity and macroscopic inflammation in both CD and UC.

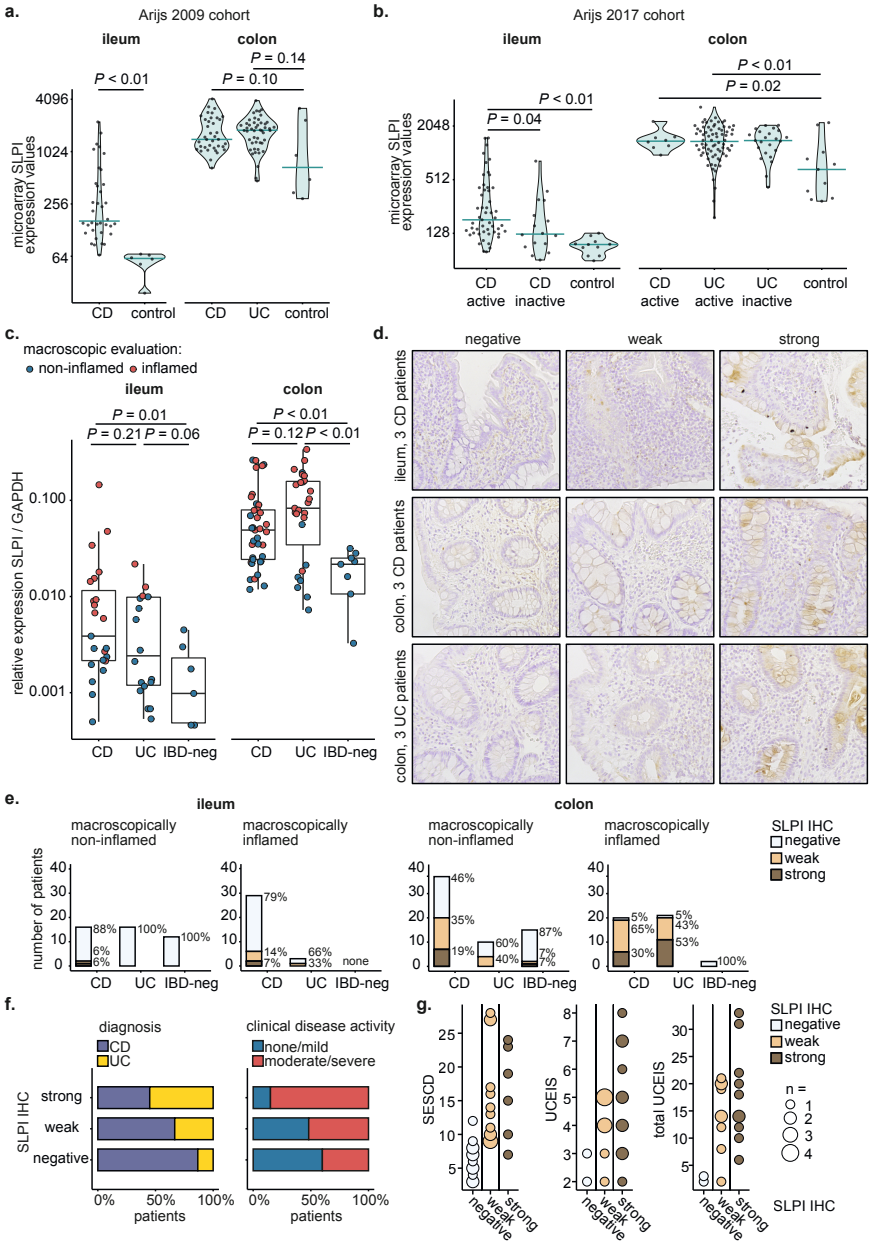

Figure 2: SLPI expression is increased in the colonic epithelium of therapy-naive pediatric CD patients and UC patients compared to IBD-negative patients

SLPI RNA expression values from microarray data of ileal and colonic biopsies from CD patients, UC patients and controls are shown in violin plots (a + b). Data is derived from Gene Expression Omnibus (GEO) datasets GSE16879 (a, Arijs 2009 cohort) and GSE75214 (b, Arijs 2017 cohort). Horizontal bars represent the median (a + b). *P*-values were calculated using the Wilcoxon rank sum test (a + b). *SLPI* mRNA expression was measured

by qPCR in macroscopically non-inflamed and macroscopically inflamed ileal and colonic biopsies from CD patients (n = 21), UC patients (n = 19) and IBD-negative patients (IBD-neg, n = 9) in the Longitudinal IBD cohort (c). Each datapoint represents one biopsy (c). The boxplots display the median and first and third quartiles (c). *P*-values were calculated using the Wilcoxon rank sum test (c). SLPI protein expression was detected by immunohistochemistry (IHC) in macroscopically non-inflamed and macroscopically inflamed ileal and colonic biopsies from CD patients (n = 41), UC patients (n = 22) and IBD-negative patients (n = 15) in the PIBD-SETQuality subcohort (d + e). Representative images were acquired at 20x magnification (d). The intensity of epithelial SLPI staining was scored in a semi-quantitative manner and the distribution of the maximum SLPI scores per macroscopically non-inflamed and inflamed ileum and colon is shown (e). The percentage of patients with each SLPI IHC score is indicated per group (e). Clinical characteristics are plotted per SLPI IHC score group using the SLPI IHC score for the colonic biopsy with the highest modified Global Histological disease Activity Score (GHAS) per patient (f + g). Percentages of patients with CD (n = 40) versus UC (n = 22) and percentages of patients with none or mild disease activity (n = 25) versus patients with moderate to severe disease activity (n = 37) are shown (f). The Simple Endoscopic Score for Crohn Disease (SES-CD) is shown for CD patients with a complete endoscopy (n = 31) (g). The UC Endoscopic Index of Severity (UCEIS) is shown for all UC patients (n = 22) (g). The 'total UCEIS' (the sum of the UCEIS of each colonic segment) is shown for UC patients with a complete endoscopy (n = 21) (g). The size of the circles represents the number of patients (g).

Colonic epithelial SLPI expression is associated with histological disease activity and infiltration of neutrophils

As we found that epithelial SLPI expression was mainly increased in macroscopically inflamed regions of the colon of IBD patients, we questioned which immune processes underlie this association. Microscopic disease activity as assessed using the modified GHAS significantly associated with high SLPI scores in colonic biopsies from CD patients, UC patients and IBD-negative patients (Kruskal-Wallis rank sum test; $P < 0.01$; Figure 3a). To assess whether microscopic disease activity is due to neutrophil infiltration, we assessed the relationship between SLPI and the activity GHAS, which is determined by the presence of neutrophils in the lamina propria and the epithelium, epithelial damage and erosions or ulcers. Microscopic disease activity as assessed by the activity GHAS significantly associated with high SLPI scores in colonic biopsies (Kruskal-Wallis rank sum test; $P < 0.01$; Figure 3a). These findings argue that colonic epithelial SLPI expression in IBD could be related to infiltration of neutrophils.

To answer whether SLPI expression is related to the presence of neutrophils, we performed immunohistochemistry for calprotectin (S100A8/S100A9 heterodimer) on colonic biopsies from CD patients, UC patients and IBD-negative patients. Fecal calprotectin concentrations are known to reflect the presence of neutrophils in the intestine and predict disease relapse in IBD (56). We observed clusters of calprotectin-positive cells in a subset of the colonic biopsies (Figure 3b). The number of calprotectin-positive cells significantly associated with high SLPI scores in colonic biopsies from CD patients, UC patients and IBD-negative patients (Fisher's exact test; $P < 0.01$; Figure 3c). To visualize the relationship between SLPI expression, diagnosis, the number of

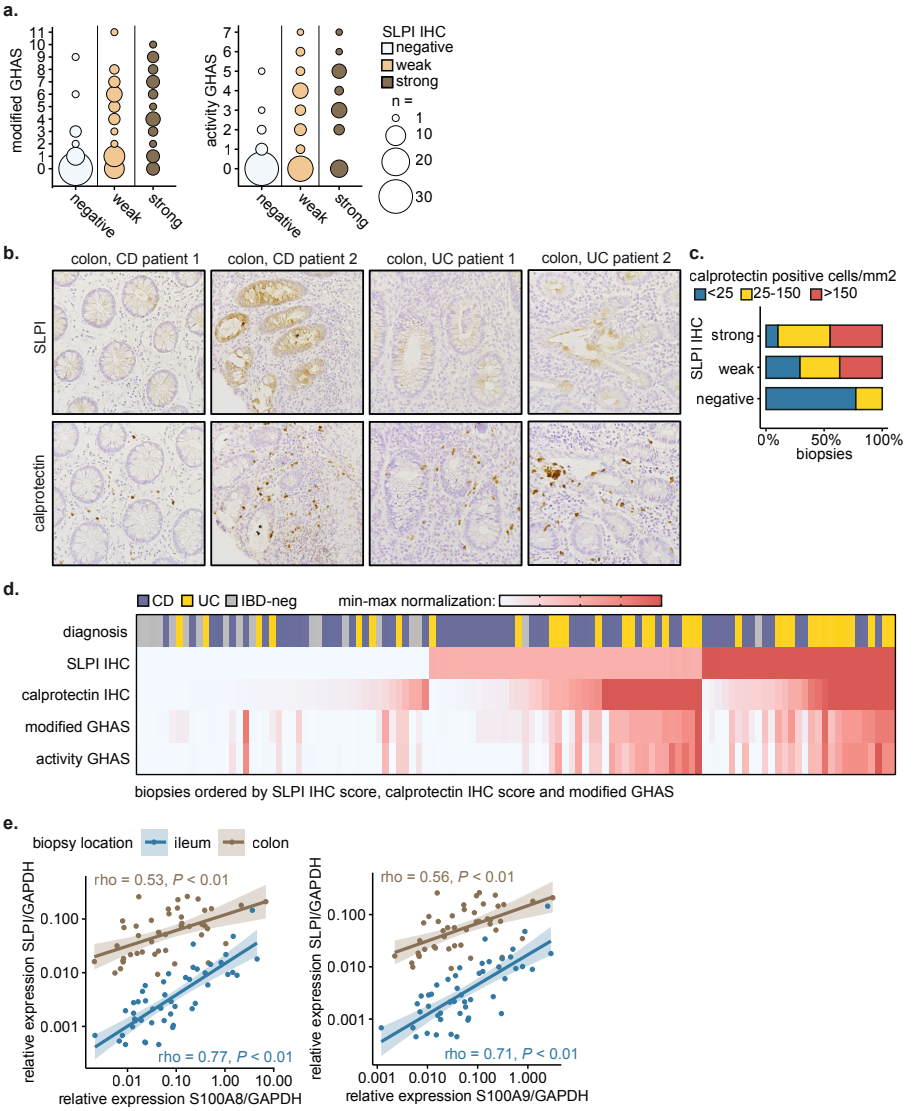

Figure 3: Colonic epithelial SLPI expression is associated with histological disease activity and with infiltration by calprotectin-positive cells

H&E-stained sections of colonic biopsies from CD patients (n = 41), UC patients (n = 22) and IBD-negative (IBD-neg) patients (n = 15) in the PIBD-SETQuality subcohort were scored using the modified Global Histological disease Activity Score (modified GHAS), including the subscore 'activity GHAS' (a). The GHAS scores are shown for the three groups of SLPI immunohistochemistry (IHC) scores (a). The size of the circles represents the number of biopsies (total n = 114 biopsies) (a). Calprotectin was detected using IHC in colonic biopsies from CD patients, UC patients and IBD-negative patients in the PIBD-SETQuality subcohort (b + c). Representative images of calprotectin IHC and SLPI IHC are shown for colonic biopsies from two CD patients and two UC patients (b). Images were acquired at 20x magnification (b). The number of calprotectin-positive cells was counted per mm^2 with a maximum of 200, and was grouped into three

categories (c). Percentages of calprotectin scores are shown per colonic SLPI IHC score (total n = 114 biopsies) (c). The relationship between the different histological scores as described above is shown in a heatmap for all colonic biopsies (n = 114) from CD patients (n = 40), UC patients (n = 22) and IBD-negative patients (n = 15) in the PIBD-SETQuality subcohort (d). Biopsies were ordered by SLPI IHC score, then by the number of calprotectin-positive cells per mm^2 with a maximum of 200 ('calprotectin IHC') and then by the modified GHAS (d). For each score, the data is normalized to the minimum and maximum within that score and the lowest score is displayed as light blue and the highest score as red (d). *SLPI, S100A8* and *S100A9* mRNA expression were measured by qPCR in ileal and colonic biopsies from CD patients (n = 21), UC patients (n = 19) and IBD-negative patients (IBD-neg, n = 9) in the Longitudinal IBD cohort (e). Each datapoint represents one biopsy (e). Spearman's rank correlation coefficient was used to assess the relationship between *SLPI* and *S100A8* mRNA expression and between *SLPI* and *S100A9* mRNA expression in both ileal and colonic biopsies (e).

calprotectin-positive cells and microscopic disease activity, we generated a heatmap of the histological data from all colonic biopsies (Figure 3d). These data show that high colonic epithelial SLPI protein expression, as detected with immunohistochemistry, is associated with microscopically active disease and a high number of calprotectin-positive cells.

To further assess the relationship between SLPI and calprotectin, we measured *SLPI* mRNA, *S100A8* mRNA and *S100A9* mRNA in ileal and colonic biopsies from CD patients, UC patients and IBD-negative patients in the Longitudinal IBD cohort. *SLPI* mRNA significantly correlated with *S100A8* mRNA and *S100A9* mRNA in both ileal biopsies and colonic biopsies (Figure 3e). Together, these data demonstrate that colonic epithelial SLPI expression is associated with neutrophil infiltration.

RNA sequencing reveals enrichment for immune activation with neutrophil and T-cell infiltration in the colon of IBD patients with high colonic epithelial SLPI expression

As neutrophils are a heterogeneous cell population with highly varying immune activity, we questioned whether the neutrophils that co-localize with high epithelial SLPI expression had a particular immune profile and what other cellular networks they may cooperate with. Thereto, we performed RNA sequencing on paired biopsies from the same patients for whom we had performed immunohistochemical detection of SLPI. For each patient, one ileal and one colonic biopsy corresponding with the location of the microscopically most affected ileal and colonic biopsy were included (Figure 4a). We categorized patients with high epithelial SLPI protein expression ($SLPI^{high}$ group) and with low epithelial SLPI protein expression ($SLPI^{low}$ group) according to their SLPI immunohistochemistry score and separated ileum from colon (Figure 4a). Based on ileal SLPI protein expression on histology, only 7 patients were categorized in the $SLPI^{high}$ group, versus 36 in the $SLPI^{low}$ group (Figure 4b). As anticipated, *SLPI* mRNA expression in these ileum biopsies was not different between $SLPI^{high}$ and

SLPI^{low} groups (Figure 4b). In contrast, in colonic biopsies, *SLPI* mRNA expression was significantly higher in patients of the SLPI^{high} group compared to the SLPI^{low} group categorized according to colonic SLPI protein expression on histology (Figure 4b). In the colon 559 genes were differentially expressed between the SLPI^{high} and SLPI^{low} groups (487 upregulated and 72 downregulated), whereas in the ileum only 5 genes were differentially expressed between the two groups (all 5 upregulated) (Wald test with Benjamini-Hochberg correction; log2 fold change < -1 or > 1 and adjusted *P*-value < 0.05). These data demonstrate that histological detection of SLPI protein expression in colon discriminates two groups of IBD patients with distinct gene expression profiles in the most affected colonic region. Therefore, we focused on colonic SLPI expression in our next analyses. Supporting the observation that SLPI is mainly expressed by goblet cells, expression of genes coding for mucins was higher in colonic biopsies from patients in the SLPI^{high} group compared to the SLPI^{low} group (Supplementary figure 3).

To identify which pathways are associated with high colonic SLPI expression and neutrophil infiltration we performed gene set enrichment analysis (GSEA) on expression profiles from colonic biopsies of patients with a high SLPI immunohistochemistry score versus a low immunohistochemistry score. This revealed significant enrichment for 26 out of 50 hallmark pathways in SLPI^{high} patients. The top 15 upregulated pathways (Figure 4c) contained many immune processes including TNF-α signaling via NF-κB, epithelial mesenchymal transition and an interferon-γ response (Figure 4c-f). In particular, amongst highly upregulated genes in the 'TNF-α signaling via NF-κB' hallmark pathway are the neutrophil attracting chemokines *CXCL1, CXCL3* and *CXCL6*, the autoinflammatory cytokine *IL1B*, the pattern recognition receptor *Toll-like receptor 2* (*TLR2*) and *PTGS2*, the gene encoding cyclooxygenase-2 involved in prostaglandin synthesis. In keeping with the strong neutrophil infiltration on histology, gene expression in biopsies from SLPI^{high} patients not only showed a signature of neutrophil infiltration, but also functional activation, illustrated by transcription of the genes *OSM*, involved in stromal cell activation, *MMP9*, involved in collagen degradation, the antimicrobial proteins calprotectin (*S100A8/S100A9* heterodimer) and Lipocalin-2 (*LCN2*) and *NCF2* encoding a subunit of the NADPH complex required for microbial killing (Figure 4e + g). This is further supported by upregulation of multiple genes in the GSEA-curated 'neutrophil degranulation pathway' (57) in the SLPI^{high} group (Supplementary figure 4a).

Apart from neutrophil activation, colonic biopsies from patients in the SLPI^{high} group versus the SLPI^{low} group were enriched in IL-17 signaling (Figure 4f + g). Key genes included the chemokine *CCL20* which attracts CCR6 expressing Th17 and ex-Th17 cells to the inflamed intestine (58) and *CXCL8,* the hallmark neutrophil recruiting chemokine

induced by IL-17 signaling (Figure 4f + g). In addition, the gene encoding the epithelial antimicrobial dual oxidase 2 (*Duox2)* was upregulated in the SLPI^{high} biopsies (Figure 4f + g). An increase in ileal *Duox2* expression has been associated with expansion of Proteobacteria in both CD and UC, possibly related to microbial shifts in the subgroup of IBD patients with high colonic epithelial SLPI expression (59).

Most striking was the overall signature of genes associated with therapy resistance in the SLPI^{high} patient group (10,11). This led us to analyze whether previously described expression modules related to therapy resistance were enriched in biopsies of SLPI^{high} patients (10,11). Indeed, most genes from the 'OSM-associated module' (10) that differentiate patients with primary non-responsiveness to anti-TNF therapy were upregulated in colonic biopsies from our therapy naive SLPI^{high} IBD patients (Supplementary figure 4a). IL1B, likely to be an important driver of inflammation in this module, requires activation through cleavage. Intriguingly, *caspase-1* (*CASP1*), the gene encoding the enzyme required for this activation was highly upregulated in SLPI^{high} patients (Figure 4g). In addition to the "OSM module", the published "neutrophil module" described to associate with therapy nonresponse (11) was upregulated in colonic biopsies from patients in the SLPI^{high} group (Supplementary figure 4c).

Taken together, colonic epithelial SLPI expression associates with strong immune activation characterized by neutrophil activation and IL-17 signaling in IBD. In addition, genes associated with therapy nonresponse are enriched in the colon of IBD patients with high epithelial SLPI expression.

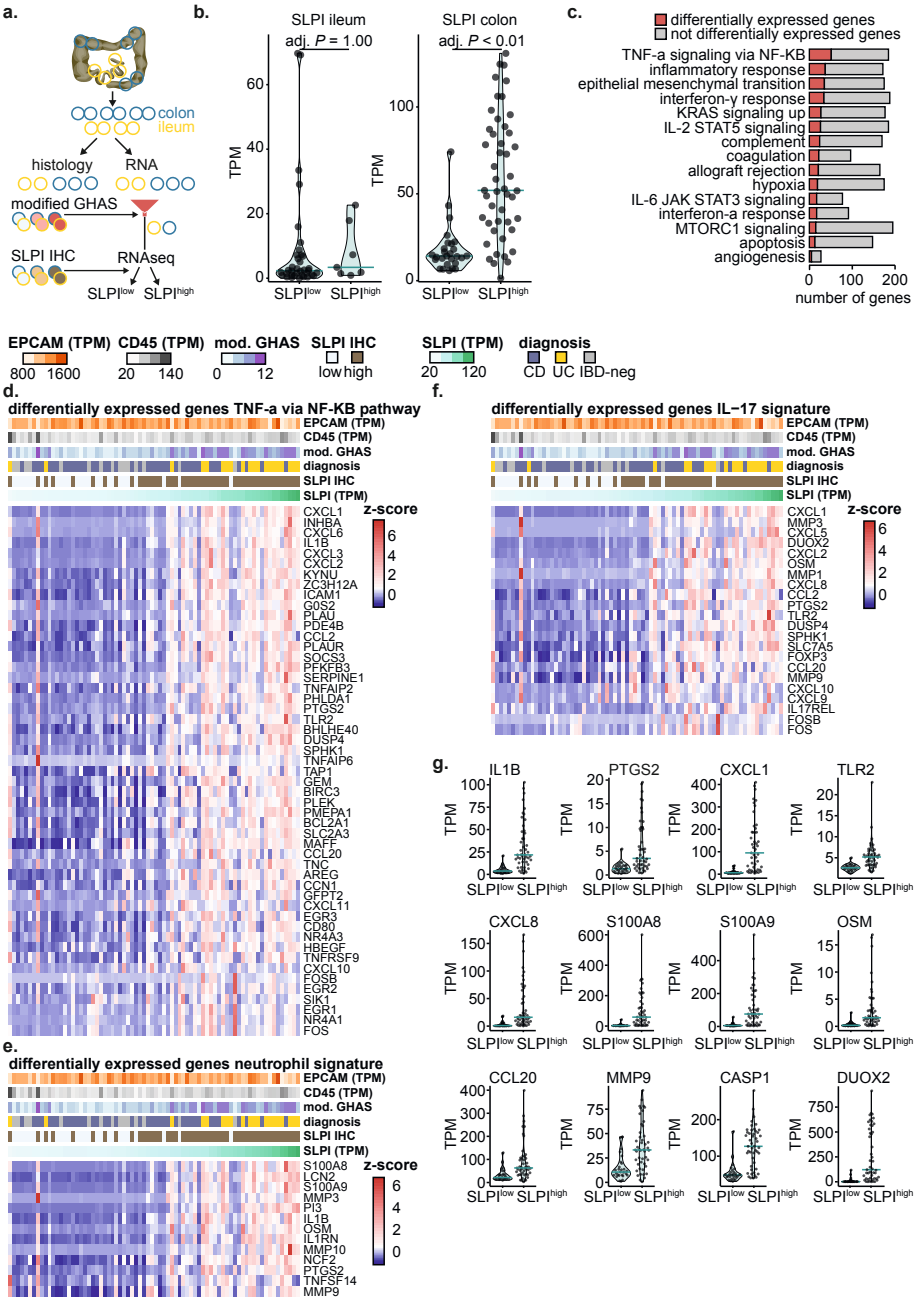

Figure 4: RNA sequencing reveals enrichment for immune activation with neutrophil and T-cell infiltration in the paired colonic biopsy from patients with high SLPI immunohistochemistry score

Two adjacent (paired) biopsies were collected from multiple ileal and colonic regions during endoscopy of therapy-naive CD patients, UC patients and IBD-negative (IBD-neg) patients in the PIBD-SETQuality subcohort (a). From the two paired biopsies, one biopsy was used for histological analysis including the modified Global Histological disease Activity Score (modified GHAS), which was used to define the most affected ileal and colonic region per patient. SLPI immunohistochemistry (IHC) scores were used to group patients as $SLPI^{low}$ (negative IHC) or $SLPI^{high}$ (weak/strong IHC). RNA sequencing was performed on the paired biopsy from the most affected ileal and colonic region per patient: ileal biopsies $SLPI^{low}$-group: CD patients (n = 24), IBD-negative patients (n = 12); ileal biopsies $SLPI^{high}$-group: CD patients (n = 7); colonic biopsies $SLPI^{low}$-group: CD patients (n = 13), UC patients (n = 1), IBD-negative patients (n = 13); colonic biopsies $SLPI^{high}$-group: CD patients (n = 26), UC patients (n = 19), IBD-negative patients (n = 2). Gene expression was compared between the $SLPI^{high}$-group and $SLPI^{low}$-group using DESeq2 followed by Benjamini-Hochberg correction of *P*-values and genes were considered differentially expressed if the log2 fold change was > 1 or < -1 and the adjusted *P*-value < 0.05 in all analyses (c + d + e + f). Violin plots of Transcripts Per Kilobase Million (TPM)-values of *SLPI* RNA are shown for ileal and colonic biopsies from the $SLPI^{low}$-group and $SLPI^{high}$-group (b). Gene Set Enrichment Analysis (GSEA) was performed and the top 15 of the 26 upregulated hallmark pathways based on DESeq2 are shown (c). Genes differentially expressed in colonic biopsies in the $SLPI^{high}$-group versus $SLPI^{low}$-group are shown in red (c). The pathways are ranked according to the number of differentially expressed genes with the highest number on top (c). The heatmaps show z-scored TPM-values of genes differentially expressed in colonic biopsies from patients in the $SLPI^{high}$-group versus $SLPI^{low}$-group (d + e + f). The genes are ranked according to significance (smallest *P*-value on top) and the samples are ranked according to SLPI TPM-values (highest TPM-value on the right) (d + e + f). The heatmaps show on top *EPCAM* and *CD45* TPM-values, SLPI immunohistochemistry score and modified GHAS score from the paired biopsy, and the diagnosis (d + e + f). The gene set 'TNF-α via NF-κB pathway' contains 51 upregulated genes and was derived from the 'hallmark TNFA signaling via NFKB' pathway (M5890) (60,61) (d). The gene set for the 'neutrophil signature' contains neutrophil products and was selected by the authors (e). The gene set for the 'IL-17 signature' contains 22 upregulated genes and was compiled using the following sources: the 'IL-17 Family Signaling Pathways SuperPath' from PathCards (62); the C2 curated gene sets 'AUJLA_IL22_AND_IL17A_SIGNALING' (M6335) (63), 'BIOCARTA_IL17_PATHWAY' (M19422) (64), REACTOME_INTERLEUKIN_17_SIGNALING' (M27382; R-HSA-448424) (57), 'WP_IL17_SIGNALING_PATHWAY' (M39560) (65); and the C5 collection Gene Ontology gene sets (60) 'GOBP_INTERLEUKIN_17_PRODUCTION (M23117), 'GOBP_POSITIVE_REGULATION_OF_INTERLEUKIN_17_PRODUCTION' (M11373), 'GOBP_RESPONSE_TO_INTERLEUKIN_17' (M23117) and 'GOMF_INTERLEUKIN_17_RECEPTOR_ACTIVITY' (M26514) (60,61,66,67) (f). Violin plots of TPM-values of representative differentially expressed genes in colonic biopsies from the $SLPI^{high}$-group versus the $SLPI^{low}$-group are shown (g).

IL-17A production is increased in the colon and peripheral blood of IBD patients with high colonic epithelial SLPI expression

As we observed enrichment in IL-17 signaling in colonic biopsies from patients with high epithelial SLPI expression, we questioned whether Th17 lymphocytes were increased in these biopsies. Therefore, we first estimated the number of mononuclear cells in the lamina propria of colonic biopsies from CD patients, UC patients and IBD-negative patients (Figure 5a + b). The number of mononuclear cells in the lamina propria was higher in colonic biopsies with high epithelial SLPI expression (Fisher's exact test, $P < 0.01$; Figure 5a + b).

As these mononuclear cells could be monocytes, macrophages and lymphocytes we next performed immunohistochemistry for IL-17 to detect IL-17-expressing lymphocytes. IL-17-positive cells were increased in a subset of biopsies, especially in biopsies with high epithelial SLPI expression and many calprotectin-positive cells (Figure 5a + c). The number of IL-17 positive cells was significantly associated with colonic epithelial SLPI protein expression (Fisher's exact test, $P < 0.01$) (Figure 5c).

To further explore the association between colonic epithelial SLPI protein expression and immune activation in IBD, we measured protein concentrations in plasma from CD patients, UC patients and IBD-negative patients. We again compared patients with high epithelial SLPI protein expression ($SLPI^{high}$ group) in the most affected colonic biopsy with patients with low epithelial SLPI protein expression ($SLPI^{low}$ group) as determined by immunohistochemistry. Strikingly, colonic epithelial SLPI expression was associated with a Th17-like immune protein profile in plasma, with the concentrations of Th17-associated cytokines IL-17A, CCL20 and IL-24 significantly increased in the plasma of patients in the $SLPI^{high}$ group versus the $SLPI^{low}$ group (Figure 5d + e + f). IL-24 is produced by Th17 lymphocytes in response to IL-17A, as autocrine negative feedback (68). Of note, IFN-γ protein concentrations were also significantly increased in the plasma of patients with high colonic epithelial SLPI expression, but with a lower fold change compared to IL-17A, suggesting a stronger association between epithelial SLPI expression and a Th17 response (Figure 5d + e + f). These data show that high colonic epithelial SLPI expression is associated with a Th17 in the peripheral blood of IBD patients.

As it has been shown that IL-17A enhances SLPI expression in human lung epithelial cells (69), we next questioned whether IL-17A enhances SLPI expression in the intestinal epithelium as well. Therefore, we stimulated the human epithelial cell line TR146 with IL-17A and cytokines known to induce SLPI in these cells (13). As expected, IFN-γ significantly downregulated *SLPI* mRNA expression in buccal epithelial cells (Figure 5g). Interestingly, IL-17A significantly upregulated *SLPI* mRNA expression (Figure 5g). In addition, IL-17A stimulation increased SLPI protein production (Figure 5h). In conclusion, IL-17A enhances SLPI expression in human buccal epithelial cells, suggesting that colonic epithelial SLPI protein expression in IBD reflects high production of IL-17A. As IL-17A also promotes recruitment of neutrophils to the tissue (70), our data argue that IL-17A enhances both epithelial SLPI expression and the accumulation of neutrophils in the colon of IBD patients.

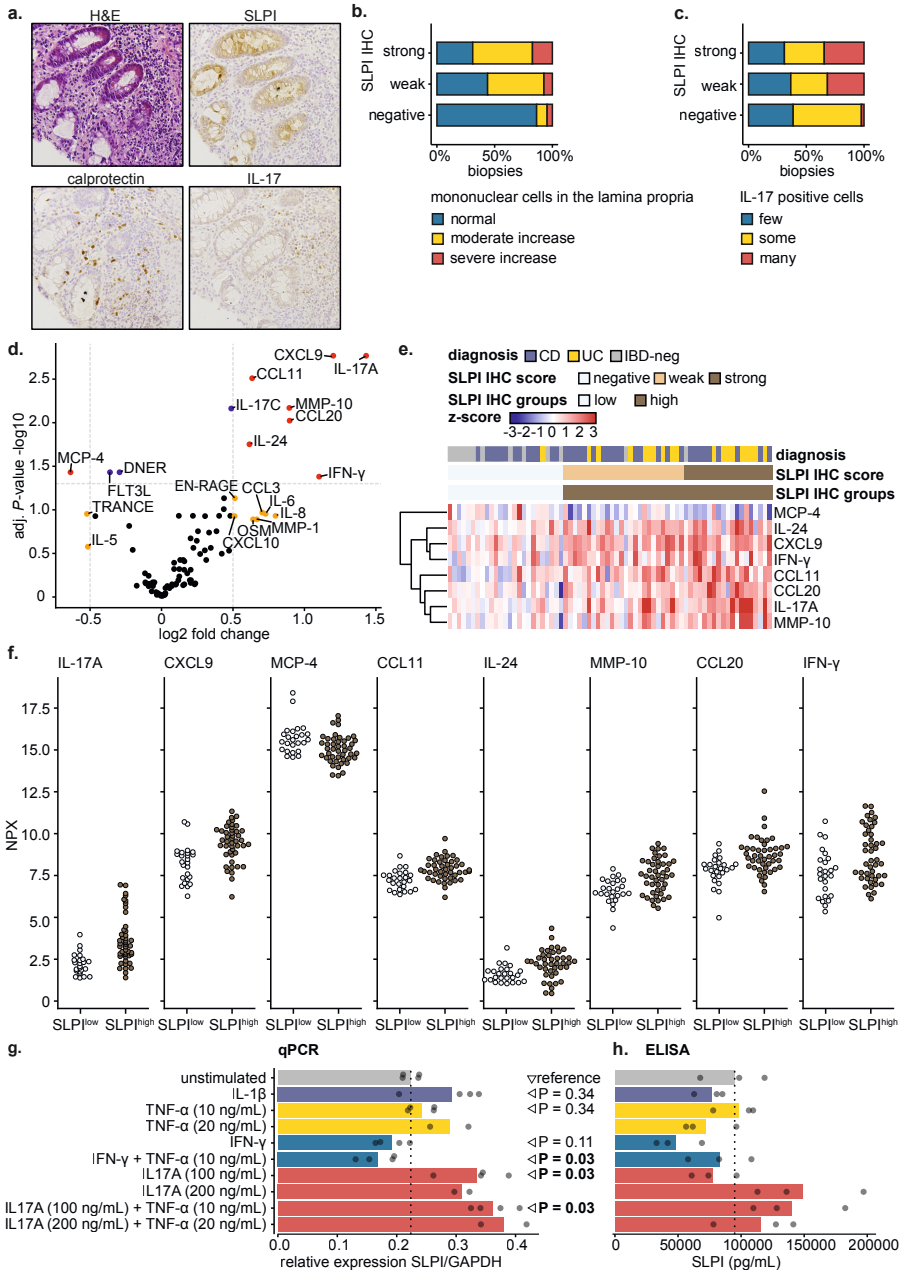

Figure 5: Colonic epithelial SLPI protein expression is associated with IL-17 positive cells in the tissue and IL-17A production in the peripheral blood

H&E staining and immunohistochemistry (IHC) was performed for SLPI, calprotectin and IL-17 on colonic biopsies from CD patients (n = 41), UCpatients (n = 22) and IBD-negative (IBD-neg) patients (n = 15) in the PIBD-SETQuality subcohort (a). Representative images of H&E staining, SLPI IHC, calprotectin IHC and IL-17 IHC of a colonic biopsy from a CD patient were acquired at 20x magnification (a). The Global Histological disease Activity Score (GHAS) parameter 'infiltration by mononuclear cells in the lamina propria' was scored on H&E-stained sections and percentages of these scores are shown for the three groups of SLPI IHC scores (total n = 114 biopsies) (b). The number of IL-17-positive cells in the lamina propria was estimated and grouped into three categories and percentages of IL-17 scores are shown for the three groups of SLPI IHC scores (total n = 114 biopsies) (c). Protein expression in plasma was measured using Olink® Proximity Extension Assay (d + e + f). SLPI IHC scores were used to group patients as $SLPI^{low}$ (negative IHC, n = 25) or $SLPI^{high}$ (weak/strong IHC, n = 45) (d + e + f). Protein expression in plasma was compared between the two groups using independent t-tests with Benjamini-Hochberg correction on Normalized Protein Expression (NPX)-values (d + e + f). Differentially expressed proteins (log2 fold change < -0.5 or > 0.5 and adjusted *P*-value < 0.05) in the $SLPI^{high}$ group compared to the $SLPI^{low}$ group are shown in red in a volcano plot (d). Blue dots represent proteins with a log2 fold change ≥ -0.5 and ≤ 0.5 and adjusted P-value < 0.05; yellow dots represent proteins with a log2 fold change < -0.5 or > 0.5 and adjusted *P*-value ≥ 0.05; and black dots represent proteins with a log2 fold change ≥ -0.5 and ≤ 0.5 and adjusted *P*-value ≥ 0.05 (d). Samples were clustered based on the eight differentially expressed proteins using hierarchical clustering with Ward's method (e). Clustering was applied on healthy control-adjusted Z-scores by first subtracting the average NPX value of 43 healthy control (not shown) and IBD-negative patients and subsequently dividing by the standard deviation (e). Patients were ordered based on SLPI IHC score (e). NPX-values for differentially expressed proteins between the $SLPI^{high}$ group and the $SLPI^{low}$ group are shown in swarm plots (f). TR146 buccal epithelial cells were cultured and starved from serum for 4-24 hours (g) or 24 hours (h) and subsequently stimulated with IL-1β (10 ng/ml), TNF-α (10 or 20 ng/mL), IFN-γ (25 ng/mL), IL-17A (100 or 200 ng/mL) or left unstimulated for 16 hours. Expression of *SLPI* mRNA expression was measured by qPCR (g) and production of SLPI protein was measured in the supernatant by ELISA (h). Bars represent the mean of multiple culture wells (g + h). Relative expression of *SLPI* mRNA was compared between unstimulated cells and stimulated cells using the Wilcoxon rank sum test (g).

DISCUSSION

IBD is a heterogenous disease and identification of distinct disease subtypes is needed to improve therapy responses. We anticipated that histological characterization of intestinal tissue from IBD patients has the potential to inform on the underlying immune processes. Here we demonstrate that immunohistochemical detection of high expression of one single epithelial cell protein, SLPI, identifies a subgroup of IBD patients with extensive inflammatory neutrophil infiltration, increased IL-17A production and high clinical disease activity at time of diagnosis.

SLPI is a very diverse protein which prevents tissue damage by inhibiting proteases including neutrophil elastase (16), has antimicrobial activity and can inhibit NF-κB mediated inflammatory gene transcription (17-19). However, the main reason for assessing SLPI expression as a histological parameter in IBD is that SLPI expression is induced in epithelium after repetitive microbial interaction (13). Here we show that increased microbial pressure upon barrier breach further increases colonic SLPI

expression in two murine models for colitis induced by microbial translocation. In silico analyses of intestinal mRNA data from cohorts of treated adult patients with IBD demonstrated increased colonic SLPI expression compared with healthy controls. This led us to question whether quantification of SLPI protein on histology would allow to capture the disturbed microbial host immune interaction in our two independent therapy-naïve pediatric IBD cohorts. We demonstrate that SLPI is mainly produced by colonic epithelial goblet cells in macroscopically inflamed regions of the colon and to a lesser extent in the ileum. This argues that SLPI expression reflects a biological process related to ongoing inflammation in IBD. Indeed, epithelial SLPI expression was associated with microscopic disease activity in colonic biopsies from IBD patients. In particular, our data indicate that infiltration by calprotectin-positive neutrophils is related to high epithelial SLPI expression in the colon. As neutrophils contain relatively little mRNA and are vulnerable to cell death, neutrophil signaling in IBD tissues is difficult to detect. However, recently high neutrophil infiltration was associated with nonresponse in IBD (11). By scoring histological disease activity and by using immunohistochemistry, we were able to identify the relationship between epithelial SLPI expression and neutrophil infiltration.

Crucially, epithelial SLPI expression was high in the most affected colonic biopsy from a subgroup of IBD patients with high clinical disease activity, demonstrating that colonic SLPI expression informs about disease activity at the patient level. Thus, our data indicate that high colonic epithelial SLPI expression is part of a strong antimicrobial response which does not occur in all IBD patients at time of diagnosis, suggesting that this subgroup may have a distinct underlying immune dysfunction.

Indeed, we demonstrate that a particular pattern of immune activation is related to high colonic epithelial SLPI expression and neutrophilic infiltration by using RNA sequencing. GSEA analyses revealed strong enrichment for many inflammatory immune pathways involving both innate and adaptive immune responses in biopsies with high SLPI expression. Most characteristic were genes involved in neutrophil chemokinesis, tissue remodeling, host-microbiota interaction and T-cell infiltration. Strikingly, amongst the upregulated genes were many genes known to associate with therapy resistance, including OSM, a neutrophil product (10,11) and its associated module. In addition, genes of the IL-1-stromal-neutrophil interaction network, previously demonstrated to associate with therapy failure, were significantly increased (10). These data argue that quantification of the single histological parameter SLPI in colonic epithelium may discriminate a subgroup of patients with severe inflammatory neutrophilic lesions that may develop therapy resistance in later stages of their disease.

High colonic epithelial SLPI expression associated with IL-17 signaling, increased IL-17 positive cells in the colonic lamina propria and a Th17- like immune protein profile in the peripheral blood. As Th17 lymphocyte differentiation is induced by microbial host interaction (71), the observed IL-17 signaling and epithelial SLPI upregulation are likely part of the same antimicrobial response. In fact, we show that IL-17A enhances SLPI expression in an epithelial cell-line, indicating that SLPI expression can be upregulated both directly by microbial contact and indirectly via IL-17A production. This is in keeping with the mechanism of SLPI expression in airway epithelium. In a murine model, SLPI expression is upregulated by IL-17A after colonization with *Bordetella pseudohinzii*, a murine-adapted airway microbe, leading to protection from lung inflammation (69). Therefore, we hypothesize that high SLPI expression in the colonic epithelium reflects an antimicrobial response to translocated microbiota which occurs in a subgroup of IBD patients. Whether epithelial SLPI expression in the colon of IBD patients is beneficial or detrimental remains to be tested. As SLPI protects mucosal tissues against inflammation by inhibiting proteases, suppressing chemokine production and killing micro-organisms, we expect that increased SLPI production limits tissue damage.

High SLPI expression has not been previously reported in IBD. As tissues from IBD patients with dense infiltration by neutrophils are associated with epithelial cell loss (11), SLPI expression may have been overlooked in bulk RNA analyses. By detecting SLPI protein in epithelial cells by immunohistochemistry, we were able to accurately discriminate patients with high and low SLPI expression and identify SLPI as a marker of a strong microbial-host immune response in IBD.

Taken together, we demonstrate that colonic epithelial SLPI expression identifies a subgroup of IBD patients with a distinct immune response and high clinical disease activity at time of diagnosis. In the context of the clinical and immunological heterogeneity of IBD, our data warrant for longitudinal studies assessing the prognostic and predictive value of SLPI in IBD. Moreover, our findings suggest that SLPI marks a disease subtype with a distinct underlying immune pathogenesis.

ACKNOWLEDGEMENTS

We thank the patients and their parents for participating in this study. We thank Martine A. Aardoom for collection of clinical data in the initial stages of this project. We thank Irma Tindemans for help with collection of biopsies from the PIBD-SETQuality subcohort. We thank Merel A. van Pieterson and Martha A. van Gaalen for coordination of patient sampling and Brenda Bley-Folly and Lisette A. van Berkel for coordinating

the plasma proteomics experiments. We thank Tom Cupedo and Eric M. J. Bindels for collaboration to obtain the RNA sequencing data. We thank Dimitris D. Rizopoulos for support with the statistical analyses. We thank Johannes Lehmann for help with data visualization.

FUNDING

The work performed in this study was funded by the Dutch Digestive Foundation (grant registration number: Focus Project 15-17), the PIBD Network for Safety, Efficacy, Treatment and Quality improvement of care project funded by the European Commission Horizon 2020 (funding source number 668 023), the Stichting Dalijn and the collaborative TIMID project (LSHM18057-SGF) financed by the PPP allowance made available by Top Sector Life Sciences & Health to Samenwerkende Gezondheidsfondsen (SGF) to stimulate public-private partnerships and co-financing by health foundations that are part of the SGF.

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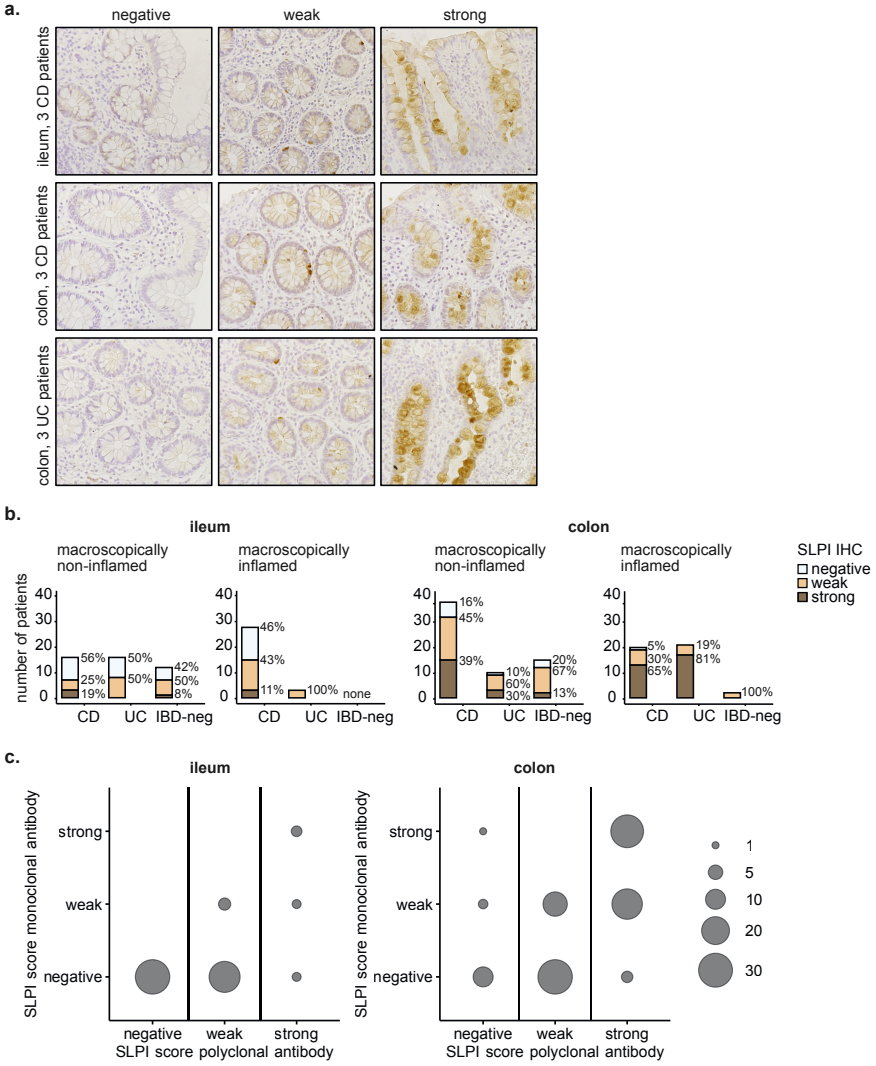


Supplementary figure 1: Relationship between SLPI immunohistochemistry scores for intestinal biopsies stained with the monoclonal and polyclonal antibody

SLPI protein expression was detected by immunohistochemistry (IHC) using the polyclonal antibody in macroscopically non-inflamed and macroscopically inflamed ileal and colonic biopsies from CD patients (n=41), UC patients (n=22) and IBD-negative patients (n=15) in the PIBD-SETQuality subcohort (a + b + c). Representative images were acquired at 20x magnification (a). The intensity of epithelial SLPI staining was scored in a semi-quantitative manner and the distribution of the maximum SLPI scores per macroscopically non-inflamed and inflamed ileum and colon is shown (b). The percentage of patients with each SLPI IHC score is indicated per group (b). The relationship between the SLPI scores of biopsies stained with the monoclonal and polyclonal antibody is shown for all ileal biopsies (n=76) and all colonic biopsies (n=114) (c). The size of the circles represents the number of biopsies.

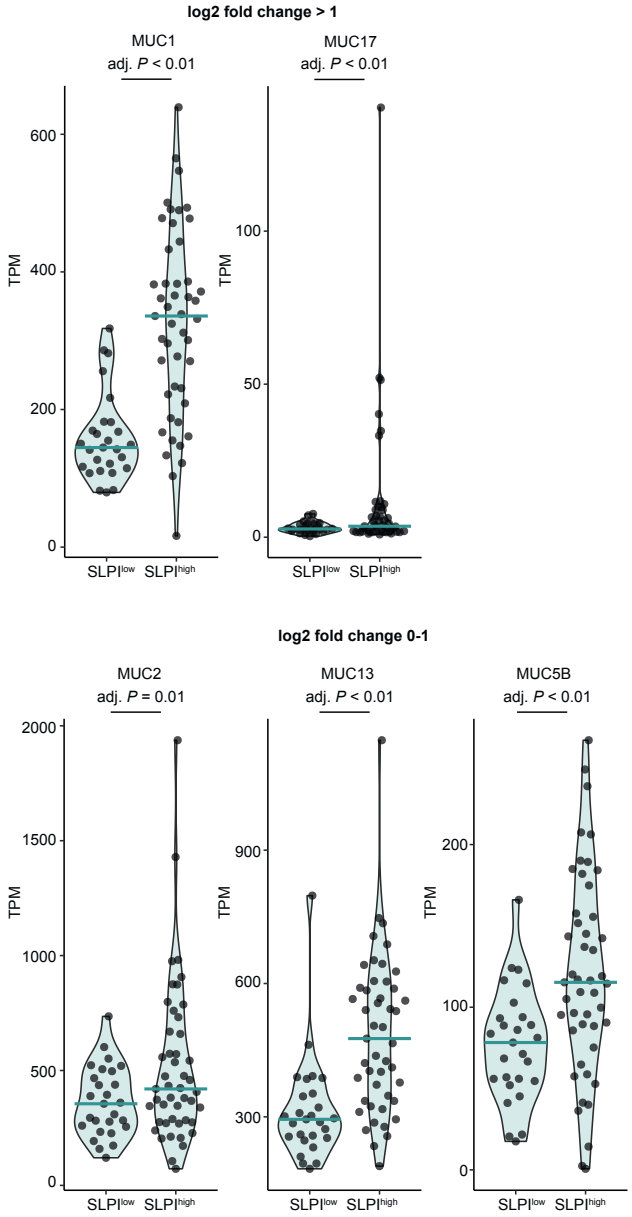
Clinical variable	SLPI IHC negative (n=15)	SLPI IHC weak (n=27)	SLPI IHC strong (n=20)	Total (n=62)	*P*-value
Age at diagnosis in years, mean (SD)	13.4 (2.5)	13.9 (2.2)	13.9 (2.5)	13.8 (2.3)	0.72[1]
Gender • female • male	8 (53%) 7 (47%)	12 (44%) 15 (56%)	10 (50%) 10 (50%)	30 (48%) 32 (52%)	0.85[2]
Diagnosis • CD • UC	13 (87%) 2 (13%)	18 (67%) 9 (33%)	9 (45%) 11 (55%)	40 (65%) 22 (35%)	**0.04[2]**
Disease activity • none or mild • moderate to severe	9 (60%) 6 (40%)	13 (48%) 14 (52%)	3 (15%) 17 (85%)	25 (40%) 37 (60%)	**0.01[2]**
SES-CD in CD patients, mean (SD)	6.4 (2.6)	15.4 (7.3)	16.3 (6.9)	12.1 (7.3)	**<0.01[1]**
UCEIS in UC patients, mean (SD)	2.5 (0.7)	4.1 (1.1)	4.9 (1.9)	4.4 (1.6)	0.14[1]
total UCEIS in UC patients, mean (SD)	2.5 (0.7)	14.4 (6.4)	18.0 (8.8)	15.0 (8.4)	0.09[1]
Disease location in CD patients • ileal • colonic • ileocolonic	12 (92%) 0 (0%) 1 (8%)	6 (33%) 2 (11%) 10 (56%)	2 (22%) 1 (11%) 6 (67%)	20 (50%) 3 (8%) 17 (43%)	**<0.01[3]**
Disease behavior • non stricturing or penetrating • stricturing • penetrating	12 (80%) 3 (20%) 0 (0%)	24 (89%) 2 (7%) 1 (4%)	0 (0%) 1 (100%) 0 (0%)	26 (79%) 6 (18%) 1 (3%)	0.46[3]
Disease extent in UC patients • ulcerative proctitis or left-sided UC • extensive UC or pancolitis	2 (100%) 0 (0%)	3 (33%) 6 (67%)	3 (27%) 8 (73%)	8 (36%) 14 (64%)	0.22[2]
Presence of granuloma in any of the biopsies from CD patients • yes • no	4 (31%) 9 (69%)	6 (33%) 12 (67%)	5 (56%) 4 (44%)	15 (38%) 25 (62%)	0.49[3]
Perianal disease • yes • no	2 (13%) 13 (87%)	2 (7%) 25 (93%)	1 (5%) 19 (95%)	5 (8%) 57 (92%)	0.71[3]

Clinical variable	SLPI IHC negative (n=15)	SLPI IHC weak (n=27)	SLPI IHC strong (n=20)	Total (n=62)	*P*-value
Fecal calprotectine in µg/g, mean (SD)	1683 (2027)	2122 (1954)	1428 (1335)	1794 (1807)	0.52[1]

[1] Kruskal-Wallis rank sum test
[2] Pearson's Chi-squared test
[3] Fisher's exact test (two-sided)

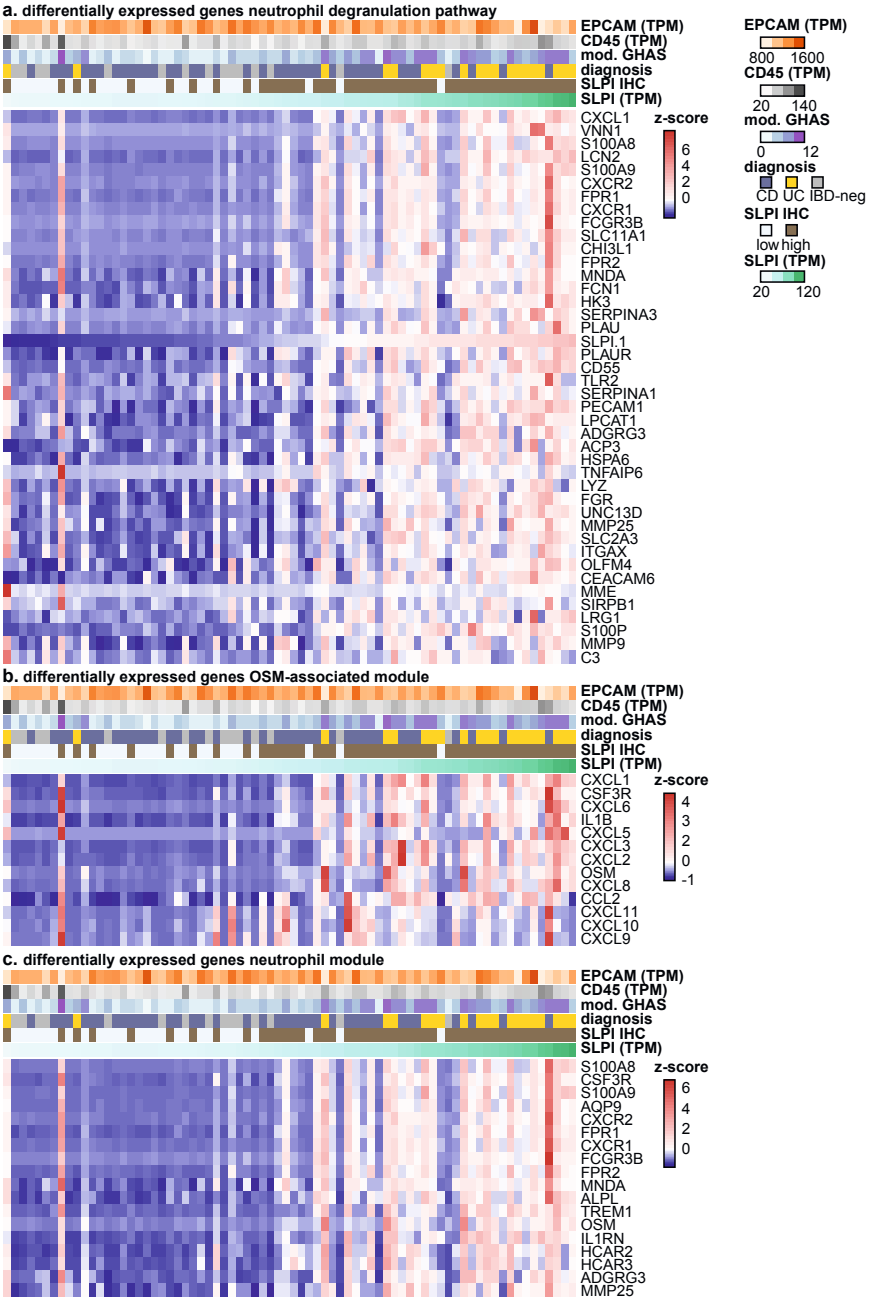
Supplementary figure 2: Clinical characteristics of IBD patients in the PIBD-SETQuality subcohort

Clinical characteristics at time of diagnostic endoscopy of CD patients and UC patients included in the PIBD-SETQuality subcohort are shown. Patients are grouped according to SLPI immunohistochemistry score for the colonic biopsy with the highest modified Global Histological disease Activity Score (GHAS). Disease location, disease behavior and disease extent are defined according to the Paris classification (30). SD = standard deviation. SES-CD = Simple Endoscopic Score for Crohn Disease. UCEIS = Ulcerative Colitis Endoscopic Index of Severity. The 'total UCEIS' is the sum of the UCEIS of each colonic segment.



Supplementary figure 3: Expression of genes encoding mucins is upregulated in the paired colonic biopsy from patients with high SLPI immunohistochemistry score

RNA sequencing was performed on the paired biopsy from the most affected colonic region per patient (as explained in figure 4a) in the PIBD-SETQuality subcohort, including CD patients, UC patients and IBD-negative (IBD-neg) patients. SLPI immunohistochemistry (IHC) scores were used to make the $SLPI^{low}$ and $SLPI^{high}$ groups. Gene expression was compared between the $SLPI^{high}$ group and $SLPI^{low}$ group using DESeq2 followed by Benjamini-Hochberg correction of *P*-values and genes were considered differentially expressed if the log2 fold change was > 1 or < -1 and the adjusted *P*-value < 0.05. Violin plots of genes encoding mucins are shown for colonic biopsies from the $SLPI^{low}$ and $SLPI^{high}$ group.



Supplementary figure 4: Neutrophil related genes are upregulated in the paired colonic biopsy from patients with high SLPI immunohistochemistry score

RNA sequencing was performed on the paired biopsy from the most affected colonic region per patient (as explained in figure 4a) in the PIBD-SETQuality subcohort, including CD patients, UC patients and IBD-negative (IBD-neg) patients. SLPI immunohistochemistry (IHC) scores were used to make the $SLPI^{low}$ and $SLPI^{high}$ groups. Gene expression was compared between the $SLPI^{high}$ group and $SLPI^{low}$ group using DESeq2 followed by Benjamini-Hochberg correction of *P*-values and genes were considered differentially expressed if the log2 fold change was > 1 or < -1 and the adjusted *P*-value < 0.05 (a + b + c). The heatmaps show z-scored Transcripts Per Kilobase Million (TPM)-values of genes differentially expressed in colonic biopsies from patients in the $SLPI^{high}$ group versus the $SLPI^{low}$ group (a + b + c). The genes are ranked according to significance (smallest *P*-value on top) and the samples are ranked according to SLPI TPM-values (highest TPM-value on the right) (a + b + c). The gene set 'neutrophil degranulation pathway' (a) was derived from the C2 curated gene set 'REACTOME_NEUTROPHIL_DEGRANULATION' (M27620) (57). The gene set 'OSM-associated module' (b) was derived from West et al. (10). The gene set 'neutrophil module' (c) was derived from Friedrich et al. (11).

CHAPTER 4

Endogenous secretory leukocyte protease inhibitor (SLPI) inhibits microbial-induced monocyte activation

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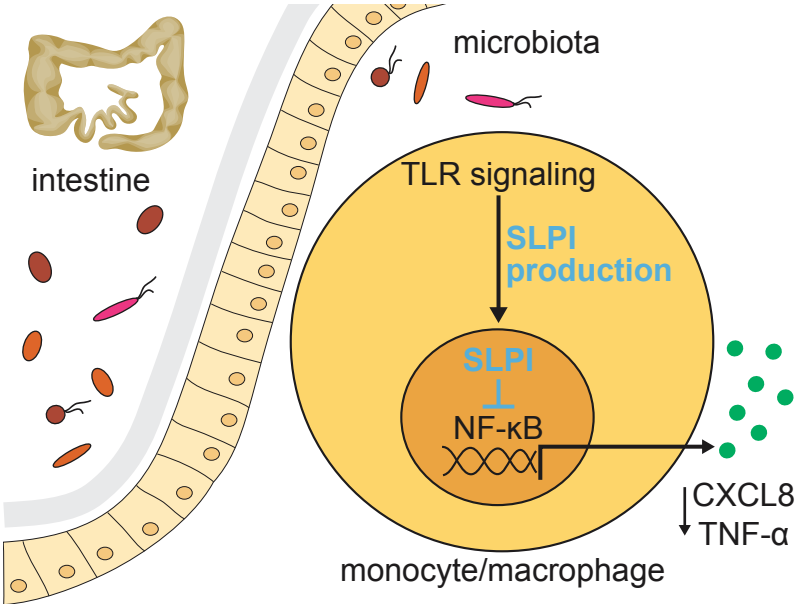
ABSTRACT

In the intestine, epithelial factors condition incoming immune cells including monocytes to adapt their threshold of activation and prevent undesired inflammation. Colonic epithelial cells express Secretory Leukocyte Protease Inhibitor (SLPI), an inhibitor of nuclear factor kappa light chain enhancer of activated B cells (NF-κB) that mediates epithelial hyporesponsiveness to microbial stimuli. Uptake of extracellular SLPI by monocytes has been proposed to inhibit monocyte activation. We questioned whether monocytes can produce SLPI and whether endogenous SLPI can inhibit monocyte activation. We demonstrate that human THP-1 monocytic cells produce SLPI and that $CD68^+$ SLPI producing cells can be detected in human intestinal lamina propria. Knockdown of SLPI in human THP-1 cells significantly increased NF-κB activation and subsequent C-X-C motif chemokine ligand 8 (CXCL8) and tumor necrosis factor alpha (TNF-α) production in response to microbial stimulation. Reconstitution of SLPI-deficient cells with either full length SLPI or SLPI lacking its signal peptide rescued inhibition of NF-κB activation and cytokine production, demonstrating that endogenous SLPI inhibits monocytic cell activation. Unexpectedly, exogenous SLPI did not inhibit CXCL8 or TNF-α production, despite efficient uptake. Our data argue that endogenous SLPI can regulate the threshold of activation in monocytes, thereby preventing activation by commensal bacteria in mucosal tissues.

GRAPHICAL ABSTRACT



We show that SLPI, an inhibitor of NF-κB, is expressed by human THP-1 monocytic cells and by CD68-expressing cells in the human intestinal lamina propria. We demonstrate that endogenous SLPI inhibits NF-κB activation and subsequent CXCL8 and TNF-α production in human THP-1 monocytic cells in response to microbial stimulation.

INTRODUCTION

Secretory leukocyte protease inhibitor (SLPI) is a protein produced by human epithelial cells (1,2), human neutrophils (3,4), human macrophages (5), human mast cells (6), and human fibroblasts (7). SLPI is an inhibitor of nuclear factor kappa light chain enhancer of activated B cells (NF-κB) signaling (8,9) and an inhibitor of serine proteases including neutrophil elastase (10). In addition, SLPI has broad antimicrobial properties (11-13). SLPI suppresses the microbiota-induced production of proinflammatory cytokines by human epithelial cells and human dendritic cells via inhibition of NF-κB signaling (14-16). Moreover, SLPI expression in dendritic cells indirectly regulates $CD4^+$ T cells in mucosa-draining lymph nodes *in vivo* (15). In consequence, *Slpi* knockout mice are more sensitive to loss of oral tolerance after feeding harmless antigens in the presence of lipopolysaccharide (LPS) compared to wild-type littermates (15). In addition, *Slpi* knockout mice are more susceptible to LPS-induced endotoxin shock compared to wild-type littermates (17). Thus, SLPI is an inhibitor of pro-inflammatory immune responses to microbial signals.

Murine macrophages have been shown to produce SLPI in response to stimulation with LPS, leading to hyporesponsiveness to LPS (18). Whether SLPI is produced by human monocytes or human macrophages is unclear. It has been reported that SLPI protein cannot be detected in untreated human myelomonocytic U937 cells and peripheral blood monocytes (9). Instead, it was demonstrated that exogenous SLPI produced by other cell types can inhibit human monocyte activation (9). Recombinant human SLPI binds to LPS extracellularly, preventing LPS-CD14 complex formation and blocking uptake of LPS by macrophages (19). Moreover, exogenous recombinant human SLPI binds to monocytes with high affinity and accesses the cytoplasm and nucleus after uptake (9,20). Once in the cytoplasm recombinant human SLPI can prevent LPS-induced degradation of the NF-κB inhibitor alpha (IκBα) in U937 cells without affecting its phosphorylation or ubiquitination (8). The subsequent inhibition of NF-κB activation leads to attenuated Toll-like receptor 2 (TLR2) and TLR4 signaling (21). In the nucleus, recombinant human SLPI competes with NF-κB p65 for NF-κB consensus-binding sites within the C-X-C motif chemokine ligand 8 (CXCL8) and tumor necrosis factor alpha (TNF-α) promoters in U937 cells (9). As such, SLPI is thought to directly prevent NF-κB p65 from binding to the NF-κB- binding sites resulting in decreased LPS-induced CXCL8 and TNF-α production (9). Thus, uptake of exogenous SLPI by monocytes from the tissue microenvironment can regulate human monocyte function.

Upon migration from the bloodstream into the intestine, monocytes adapt to the local environment to either maintain homeostasis or promote inflammation (22). In the non-

inflamed intestine, monocytes become hyporesponsive to microbial signals, thereby preventing pathogenic inflammatory responses to the commensal bacteria residing in close proximity (23,24). In the human and murine gastrointestinal tract, SLPI expression is abundant in epithelial cells (16,25,26). Previously, we showed that repetitive microbial stimulation drives SLPI expression and mediates the acquisition and maintenance of hyporesponsiveness to microbial signals in buccal and intestinal epithelial cells (16). Also *in vivo* colonization of germ-free mice with commensal microbiota elicits SLPI expression and suppresses chemokine release by colonic epithelial cells (16). In line with the dense colonization in the colon, SLPI protein expression is higher in the colonic epithelium compared to small intestinal epithelium in both human and mice (16,26). Conversely, in the small intestine, SLPI protein expression is more dominant in cells in the lamina propria (16). Therefore, the presence of SLPI protein in small intestinal lamina propria cells cannot solely be explained by uptake of exogenous SLPI released by epithelial cells. This prompted us to question whether human monocytes also produce SLPI. As SLPI regulates proinflammatory cytokine and chemokine production, we hypothesized that microbial signals induce SLPI expression in monocytes and that this endogenous SLPI regulates subsequent monocyte function in mucosal tissues.

Here we show that SLPI is expressed in human small intestinal and colonic mononuclear cells, amongst which $CD68^+$ monocytes or macrophages, from both pediatric controls and pediatric Crohn's disease patients. Using the human monocytic cell line THP-1, we show that monocytes produce SLPI which suppresses endogenous LPS-induced CXCL8 and TNF-α production via inhibition of NF-κB signaling. In contrast, exogenous recombinant human SLPI was unable to inhibit CXCL8 and TNF-α production by activated THP-1 cells, despite detectable uptake in the nucleus and cytoplasm. In conclusion, we demonstrate that endogenous SLPI significantly inhibits monocyte activation upon microbial stimulation.

MATERIALS AND METHODS

Collection of human intestinal tissue

Resection material was collected during surgery from pediatric Crohn's disease (CD) patients from a longitudinal IBD cohort. For IBD-negative controls, biopsies were collected during diagnostic endoscopy from pediatric patients suspected of having inflammatory bowel disease (IBD) but negative diagnosis as part of the PIBD Network for Safety, Efficacy, Treatment and Quality improvement of care (PIBD-SETQuality) study (27). Patients were patients < 18 years old. Both the surgeries and the endoscopies took place at the Erasmus Medical Center in Rotterdam, The Netherlands. Tissues were formalin-fixed and paraffin-embedded and 4-micrometer-thick sections were

cut and mounted on polysine adhesion glass slides (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Detection of human SLPI protein and CD68 protein in intestinal tissue by immunohistochemistry

A single staining was performed for SLPI and a double staining was performed for SLPI and CD68, expressed by monocytes/macrophages. Sections were deparaffinized in xylene and rehydrated in ethanol. Subsequently, sections were incubated in 3% H_2O_2 in phosphate buffered saline (PBS) for 20 min to quench endogenous peroxidase activity. Antigen retrieval was performed by microwave treatment in citrate buffer (10 mM, pH 6.0). Sections were blocked in Tris buffer (10 mM, pH 8.0) containing 5 mM EDTA (pH 8.0), 0.15 M NaCl, 0.25% gelatin, 0.05% Tween-20 and 10% normal human serum (AB serum; Sanquin, Amsterdam, The Netherlands) plus 10% normal rabbit serum (Jackson ImmunoResearch, West Grove, PA, USA) and for the double staining also with 10% normal horse serum (Biowest, Nuaillé, France) for one hour at room temperature. For the double staining, sections were also blocked in BLOXALL (Vector Laboratories, Burlingame, CA, USA) for 10 min to inactivate endogenous alkaline phosphatase. For both the single staining and the double staining sections were stained with a polyclonal anti-human-SLPI antibody (1 µg/mL, goat IgG, BAF1274; R&D Systems/Bio-Techne, Minneapolis, MN, USA) and only for the double staining sections were also stained with a monoclonal anti-human-CD68 antibody (0,2 mg/mL, mouse IgG1κ, clone KP1, LS-B2862; LSBio, Seattle, WA, in PBS overnight at 4 °C. As control, one section was stained with isotypes (for SLPI goat IgG, AB-108-C; R&D Systems/Bio-Techne and for CD68 mouse IgG1, HI1016; Hycult Biotech, Uden, The Netherlands) instead of primary antibodies (Supplementary figure 1a) and one section was incubated with PBS (Supplementary figure 1b: conjugate control). For the double staining, sections were incubated the next day with an alkaline phosphatase horse-anti-mouse antibody using the ImmPRESS-AP Horse Anti-Mouse IgG Polymer Detection Kit (Vector Laboratories) for 30 min at room temperature. Subsequently, an alkaline phosphatase substrate, BCIP/NBT (Vector Laboratories) was used to detect the alkaline phosphatase antibodies. For both the single staining and the double staining SLPI was detected with a biotinylated secondary rabbit-anti-goat antibody (1:500, Vector Laboratories) for one hour at room temperature followed by a complex of avidin and biotin (Vectastain ABC Elite Kit, Vector Laboratories) and 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, Zwijndrecht, The Netherlands). For the single staining sections were counterstained with hematoxylin (Vector Laboratories) for 5 seconds and for the double staining sections were counterstained with methyl green (Vector Laboratories) at 60°C for 1 min. Subsequently, sections were dehydrated and immersed in xylene and mounted in Entallan™ (Sigma-Aldrich). Images of stained

sections were digitally captured on a Leica DM5500B microscope equipped with a Leica DFC420C camera using a 20x brightfield lens (Leica 506503) and a 63x brightfield lens (Leica 506223).

Culture of THP-1 cells

THP-1 cells (ATCC® TIB-202™, Manassas, Virginia, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco™, Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal calf serum (FCS, Bodinco BV, Alkmaar, the Netherlands), 1x non-essential amino acid solution (Gibco™, Thermo Fisher Scientific) and 25 U/mL penicillin-streptomycin (Gibco™, Thermo Fisher Scientific), according to manufacturer's instructions. Stimulation of THP-1 cells with lipopolysaccharide (LPS, Sigma-Aldrich), phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich), recombinant human interferon gamma (IFN-γ, Immunotools GmbH, Friesoythe, Germany) and recombinant human SLPI (R&D; catalog number 1274-PI) was performed at concentrations as described in the figure legends.

Knockdown and reconstitution of SLPI in the human monocytic cell line THP-1 using shRNA

SLPI was knocked down in THP-1 cells using retroviral vector-based short hairpin RNA (shRNA). RNA interference was achieved using short interfering RNAs targeted against human SLPI (SMARTpool; Dharmacon RNA Technologies, Lafayette, Colorado, USA). Short interfering RNAs were subcloned into a pRSC DNA construct (a kind gift from prof. dr. R. Bernards, The Netherlands Cancer Institute, Amsterdam, The Netherlands) for stable RNA interference as described previously (16). The pRSC vector is a modification of the pRETRO-SUPER (28). THP-1 cells were transduced with virus supernatant containing shRNA specific for SLPI in the pRSC vector.

To rescue silencing of the SLPI gene, cells were transfected with a construct containing either full length SLPI (construct from Open Biosystems, Inc. Huntsville, Alabama, USA) or truncated SLPI. Truncated SLPI lacks the 75-nucleotide sequence for its signal peptide (25 amino acids) as described by others (18,29). The DNA encoding full length SLPI or truncated SLPI was subcloned into a LZRS-IRES-EGFP DNA construct. These constructs were transfected into THP-1 cells not containing any vector or already containing the pRSC constructs for SLPI RNA interference by retroviral transduction.

Cell lines:

construct
THP-1 + empty LZRS-IRES-EGFP
THP-1 + pRSC-shRNA[anti-SLPI]
THP-1 + pRSC-shRNA[anti-SLPI] + empty LZRS-IRES-EGFP
THP-1 + pRSC-shRNA[anti-SLPI] + LZRS-IRES-EGFP-truncated SLPI
THP-1 + pRSC-shRNA[anti-SLPI] + LZRS-IRES-EGFP-full length SLPI

Sequences:

siRNAs to silence SLPI:

AGTCTGTCCTCCTAAGAAA + TTTCTTAGGAGGACAGACT

full length SLPI: ATGAAGTCCAGCGGCCTCTTCCCCTTCCTGGTGCTGCTTGCCCTG
GGAACTCTGGCACCTTGGGCTGTGGAAGGCTCTGGAAAGTCCTTCAAAGCTGGAGTC
TGTCCTCCTAAGAAATCTGCCCAGTGCCTTAGATACAAGAAACCTGAGTGCCAGAG
TGACTGGCAGTGTCCAGGGAAGAAGAGATGTTGTCCTGACACTTGTGGCATCAAATGCC
TGGATCCTGTTGACACCCCAAACCCAACAAGGAGGAAGCCTGGGAAGTGCCCAGTGAC
TTATGGCCAATGTTTGATGCTTAACCCCCCCAATTTCTGTGAGATGGATGGCCAGTGCAAG
CGTGACTTGAAGTGTTGCATGGGCATGTGTGGGAAATCCTGCGTTTCCCCTGTGAAAGCTT
GATTCCTGCCATATGGAGGAGGCTCTGGAGTCCTGCTCTGTGTGGTCCAGGTCCTTTCCAC
CCTGAGACTTGGCTCCACCACTGATATCCTCCTTTGGGGAAAGGCTTGGCACACAGCAG
GCTTTCAAGAAGTGCCAGTTGATCAATGAATAAATAAACGAGCCTATTTCTCTTTGCA

truncated SLPI:

TCTGGAAAGTCCTTCAAAGCTGGAGTCTGTCCTCCTAAGAAATCTGCCCAGTGCCTTAGA
TACAAGAAACCTGAGTGCCAGAGTGACTGGCAGTGTCCAGGGAAGAAGAGATGTTGTCCT
GACACTTGTGGCATCAAATGCCTGGATCCTGTTGACACCCCAAACCCAACAAGGAGGAAG
CCTGGGAAGTGCCCAGTGACTTATGGCCAATGTTTGATGCTTAACCCCCCCAATTTCTGT
GAGATGGATGGCCAGTGCAAGCGTGACTTGAAGTGTTGCATGGGCATGTGTGGGAAATCCT
GCGTTTCCCCTGTGAAAGCTTGATTCCTGCCATATGGAGGAGGCTCTGGAGTCCTGCTCT
GTGTGGTCCAGGTCCTTTCCACCCTGAGACTTGGCTCCACCACTGATATCCTCCTTTGGG
GAAAGGCTTGGCACACAGCAGGCTTTCAAGAAGTGCCAGTTGATCAATGAATAAATA
AACGAGCCTATTTCTCTTTGCA

Retroviral transduction

To produce retrovirus, the Phoenix-AMPHO packaging cell line (a kind gift from dr. J. Meijerink, Princess Maxima center for Pediatric Oncology, Utrecht, The Netherlands) was transfected with 10 µg of either pRSC empty vector (mock control) or pRSC shSLPI DNA constructs by standard calcium phosphate transfection. Supernatant containing

virus was harvested after 48 hours and filtered before infection. THP-1 cells were cultured to a maximum of 800,000 cells per milliliter at the moment of infection. Cells and virus were spun down in a flat bottom plate for 45 minutes at 1000g at room temperature and cultured at 37 °C for 2 hours. This infection cycle was repeated 3 times. Stable transduction with pRSC constructs was achieved by culturing cells under increasing Puromycin (Gibco™, Thermo Fisher Scientific) pressure by gradually increasing the concentration of Puromycin to reach a maximum of 1 µg/mL in 21 days. Subsequently, cell lines were transduced with LZRS-IRES-EGFP empty vector or LZRS-IRES-EGFP-truncated SLPI or LZRS-IRES-EGFP-full length SLPI constructs, after which cells were FACS-sorted for EGFP expression multiple times until at least 90% of the cells was EGFP positive.

Validation

SLPI mRNA knockdown and subsequent reconstitution were validated by quantitative real time PCR as described below. In addition, SLPI knockdown and subsequent reconstitution were validated at protein level by detection of SLPI in the cell supernatant by Enzyme-Linked Immuno Sorbent Assay (ELISA) as described below.

RNA extraction and cDNA synthesis

Total RNA of THP-1 cells was extracted from cells using the NucleoSpin RNA extraction kit (Macherey-Nagel GmbH & Co. KG, Dueren, Germany) for isolation of RNA according to manufacturer's instructions. Quantity and purity of extracted RNA was assessed using a DeNovix DS-11 spectrophotometer (DeNovix Inc., DE, USA). A maximum of 1 µg mRNA was used to synthesize cDNA. cDNA was synthesized using the SensiFAST cDNA Synthesis Kit (Bioline, London, United Kingdom).

Quantitative PCR

Real-time quantitative PCR (qPCR) was performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Veenendaal, The Netherlands) using the SensiMix™ SYBR® Hi-ROX Kit (Bioline). Gene expression was analyzed in duplicate and normalized using housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Relative expression was calculated as $2^{-(\text{Ct value GAPDH} - \text{Ct value gene of interest})}$. Primers used are shown below.

human gene	forward primer	reverse primer
GAPDH	GTCGGAGTCAACGGATT	AAGCTTCCCGTTCTCAG
SLPI	TCCAGGGAAGAAGAGATGT	TGCCCATGCAACACTT
TNFAIP3	TGGCACAACTCATCTCATC	CCCTGCTCGCTGTTTT
SIGIRR	GCAGACCCATCTTCATCA	ACTCGGCCTCGAAGAA
TOLLIP	AGGGCGTTGGCTATGT	CACCTCCTGGTCCATGT

Isolation of nuclear and cytoplasmic fractions

The nuclear and cytoplasmic fractions of THP-1 cells were extracted by a high-salt extraction method using the Nuclear Extract Kit (Active Motif, La Hulpe, Belgium) according to manufacturer's instructions. In short, cells were collected in ice-cold PBS with phosphatase inhibitors to limit further protein modifications. Subsequently, cells were lysed using a hypotonic buffer and detergent. The supernatant containing the cytoplasmic fraction was collected. Subsequently, in order to collect the nuclear fraction, the nuclei in the pellet were lysed and solubilized.

For detection of SLPI in nuclear and cytoplasmic fractions after addition of recombinant human SLPI, nuclear and cytoplasmic fractions were isolated according to the method described by Taggart et al. (8), with the following adjustments. For isolation of the cytoplasmic fraction, lysis was performed for 20 minutes. In addition, for isolation of the nuclear fraction, 50 µg/mL deoxyribonuclease (Sigma-Aldrich) was added to the lysis buffer and lysis was performed for 15 minutes at 37 °C and subsequently deactivated with 0.05M EDTA. SLPI protein was measured by ELISA as described below. Total protein concentrations were measured using the Bradford method (30).

Detection of SLPI, CXCL8 and TNF-α protein

For detection of SLPI protein, a 96-wells high binding surface flat-bottom plate (Corning®; 9018) was coated with 0.5 µg/mL monoclonal anti-human SLPI antibody (R&D; MAB1274, clone 20409,) in PBS overnight at 4°C. Washing was performed with 0,05% TWEEN 20 (Sigma-Aldrich) in PBS. The plate was blocked with 10% FCS (Bodinco B.V.) in PBS for 1 hour at room temperature. Subsequently, samples were incubated for 2 hours at room temperature. Recombinant human SLPI (R&D; 1274-PI) was used to generate a standard curve. To detect SLPI, the plate was incubated with 0.4 µg/mL biotinylated polyclonal goat antibody against human SLPI (R&D, BAF1274) for 1 hour at room temperature and subsequently incubated with 1:5000 horseradish peroxidase (HRP)-conjugated streptavidin (BD Biosciences; 554066) in the dark at room temperature for approximately 1 hour. 3, 3',5 ,5'-Tetramethylbenzidine Liquid Substrate (Sigma-Aldrich; T4444) was used for detection of the HRP. The reaction was stopped using 1M H_3PO_4. Signal was detected at 450+570 nanometer on a VersaMax reader (Molecular Devices, San Jose, CA, USA).

Human CXCL8 and TNF-α protein were detected using a BD OptEIA™ set (BD Biosciences, San Jose, California, USA; catalog numbers 555244 and 555212 respectively) according to manufacturer's instructions.

Detection of phosphorylated NF-κB and phosphorylated IκBα

Phosphorylated NF-κB was detected in the nuclear fraction and phosphorylated IκBα in the cytoplasmic fraction of THP-1 cells by western blot. As loading controls, human Origin Recognition Complex subunit 2 (ORC2) was detected in the nuclear fraction and human β-tubulin in the cytoplasmic fraction. Proteins were separated by 12.5% SDS-PAGE and transferred to a 0.2 µm nitrocellulose membrane. Tris-buffered saline (TBS) with 0.1% TWEEN 20 (Sigma-Aldrich) and 5% non-fat dry milk or 5% bovine serum albumin (BSA) for detection of phosphorylated NF-κB was used as blocking buffer. Primary and secondary antibodies used for detection are shown below. Incubation with the primary antibody was performed overnight at 4 °C with rotation. Incubation with the secondary antibody was performed for 1 hour at room temperature with rotation in the dark. Signal was detected using an Odyssey infrared imaging system (LI-COR Biotechnology GmbH, Bad Homburg, Germany). The intensity of the bands was quantified using the Odyssey Application Software. Plotted intensities are relative to the intensity of the corresponding loading controls.

human protein	primary antibody	secondary antibody
phosphorylated NF-κB	1:500 rabbit anti-phospho-NF-κB p65 (Ser536) polyclonal antibody (Cell Signaling Technology Europe, B.V., Leiden, The Netherlands; 3031)	goat-anti-rabbit IRDye® 800CW (LI-COR; 926-32211)
ORC2	1:2000 rabbit anti-ORC2 polyclonal antibody (BD Pharmingen™, BD Biosciences; 559266)	goat-anti-rabbit IRDye® 680RD (LI-COR; 926-68071)
phosphorylated IκBα	1:500 mouse monoclonal anti-phospho-IκBα (Ser32/36) antibody (Cell signaling Technology Europe B.V.; 9246)	goat-anti-mouse IRDye® 800CW (LI-COR; 926-32210)
β-tubulin	1:200 rabbit β-tubulin polyclonal antibody (H-235) (Santa Cruz Biotechnology, Dallas, Texas, USA; sc9104)	goat-anti-rabbit IRDye® 680RD (LI-COR; 926-68071)

Statistical analyses

Mean expression was compared between groups using the Wilcoxon rank sum test. Experiments representative for a number of experiments are shown, as indicated in the figure legends. All statistical analyses and visualization were performed using R version 3.5.1 (31). The package ggplot2 (32) was employed for visualization.

Ethics approval

For the collection of human intestinal tissue, all patients and parents signed informed consent. The IBD longitudinal cohort study was approved by the Medical Ethical

Committee of the Erasmus University Medical Centre-Sophia Children's Hospital Rotterdam (METC 2007-335). The PIBD-SETQuality study was approved by the Medical Ethical Committee of the Erasmus University Medical Centre-Sophia Children's Hospital Rotterdam (METC number: trial registration number NCT03571373) (27). This study does not include experiments using animals.

RESULTS

SLPI is produced by human monocytes

To assess whether human monocytes express SLPI, we searched for *SLPI* mRNA expression in single cell data available from the Human Protein Atlas project (33,34) and from the database of immune cell expression, expression quantitative trait loci, and epigenomics (DICE) project (35). These single cell RNA-sequencing data show that *SLPI* mRNA is expressed by peripheral blood classical- and non-classical- monocytes, by monocytes in the pancreas and by the monocytic cell line THP-1. We therefore measured *SLPI* mRNA expression in THP-1 cells and confirmed that unstimulated THP-1 cells express *SLPI* mRNA (Figure 1a). In addition, we detected substantial amounts of SLPI protein in the nucleus, cytoplasm and supernatant of unstimulated THP-1 cells after 24 hours of culture (Figure 1b).

SLPI is a known potent inhibitor of NF-κB signaling (8,9,21). Therefore, we hypothesized that endogenous SLPI regulates monocyte function via NF-κB inhibition. In order to gain insight in the relative contribution of SLPI as inhibitor of NF-κB, we measured mRNA expression of the other known NF-κB inhibitors TNF alpha induced protein 3 (TNFAIP3), single Ig and TIR domain containing (SIGIRR) and Toll-interacting protein (TOLLIP) in THP-1 cells. *SLPI* mRNA expression was significantly higher than the expression of the other NF-κB inhibitors in THP-1 cells, suggesting a possible role for endogenous SLPI in regulating NF-κB signaling in monocytic cells (Figure 1a).

Previously, we detected SLPI expression in cells in the lamina propria of the murine small intestine (16). Therefore, we questioned whether immune cells in the human intestine also express SLPI. Using immunohistochemistry, we detected SLPI protein expression in the cytoplasm and the nucleus of mononuclear cells in the lamina propria of colonic tissue from both patients without inflammatory bowel disease (11 out of 15 patients tested) and patients with Crohn's disease (10 out of 15 patients tested) (Figure 1c - h). A double staining for SLPI and CD68 indicated that these SLPI-positive cells are monocytes or macrophages (Figure 1c - h). Together, these data show that monocytes can express SLPI in health and disease and led us to hypothesize that endogenous SLPI expression regulates the responsiveness of monocytes to microbial signals in the intestine.

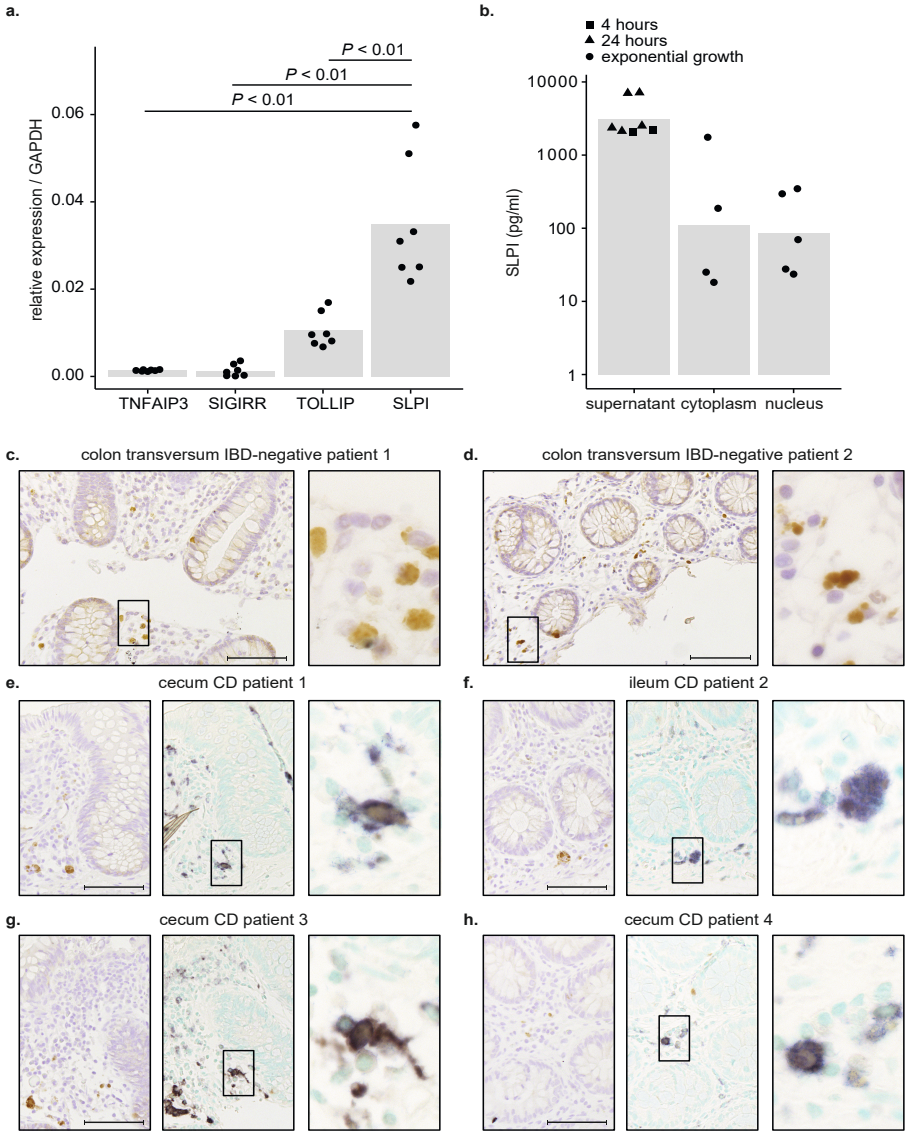


Figure 1: SLPI is produced by THP-1 cells and is expressed by mononuclear cells in the human intestinal lamina propria

(a) mRNA expression of NF-κB inhibitors *TNFAIP3*, *SIGIRR*, *TOLLIP* and *SLPI* was measured by qPCR in wildtype THP-1 cells after 24 hours of culture without stimulation.*P*-values were calculated using the Wilcoxon rank sum test. The graph combines data from two experiments and each datapoint represents a mean of two technical duplicates. Grey bars represent the mean of seven datapoints in each respective group. (b) SLPI protein expression was measured by ELISA in the cytoplasmic and nuclear fractions of wildtype THP-1 cells harvested in their exponential growth phase (black dots), and in supernatant of wildtype THP-1 cells 4 or 24 hours after refreshing medium (black squares and triangles respectively) without stimulation. The graph combines data from four experiments and each datapoint represents a single measurement for

one culture well. Grey bars represent the geometric mean of four to seven datapoints in each respective group. (c - h) SLPI protein expression was detected in lamina propria of biopsies from 11 out of 15 pediatric patients without inflammatory bowel disease (IBD-negative) (c and d) and in resection material of 10 out of 15 pediatric Crohn's disease (CD) patients (e - h; left panels) using immunohistochemistry, with a hematoxylin counterstain. Representative images are shown. In addition, SLPI protein expression (brown) and CD68 protein expression (indigo) were simultaneously detected in the same resection material from the pediatric CD patients in the next serial section using immunohistochemistry, with a methyl green counterstain (e - h; middle and right panels). Scale bars represent 100 μm (c - h). The isotype control and conjugate control for the double staining are shown in Supplementary figure 1a and 1b.

Endogenous SLPI regulates LPS-induced CXCL8 and TNF-α production by THP-1 cells

To assess whether endogenous SLPI inhibits monocyte function, we knocked down SLPI expression in THP-1 cells by retroviral transduction of short hairpin RNA. Knockdown of SLPI effectively inhibited *SLPI* mRNA expression and protein production (Figure 2a + b). SLPI-deficient THP-1 cells were more activated as demonstrated by significantly increased LPS-induced CXCL8 and TNF-α production in the supernatant of SLPI-deficient THP-1 cells compared to wildtype THP-1 cells (Figure 2c + d and Supplementary Figure 2a + b). In addition, pre-incubation with IFN-γ, which upregulates TLR expression, and subsequent LPS-stimulation increased CXCL8 and TNF-α production up to 6-fold in SLPI-deficient THP-1 cells compared to wildtype control (Figure 2c + d and Supplementary Figure 2a + b). These data demonstrate that endogenous SLPI regulates pro-inflammatory chemokine and cytokine production by monocytic cells after microbial contact.

To investigate how endogenous SLPI suppresses CXCL8 and TNF-α production in monocytic cells, we investigated the NF-κB signaling pathway in the nuclear and cytoplasmic fraction of THP-1 cells after stimulation with LPS. SLPI has been reported to suppress TLR-induced phosphorylated NF-κB accumulation in the nucleus (8,16). In the cytoplasm, SLPI inhibits TLR-induced degradation of non-phosphorylated IκBα but does not inhibit IκBα phosphorylation (8,16). Therefore, we anticipate that SLPI-deficient THP-1 cells should have both increased nuclear phosphorylated NF-κB and cytoplasmic phosphorylated IκBα, resulting in enhanced NF-κB activation. In wildtype THP-1 cells, LPS-stimulation increased phosphorylated NF-κB in the nucleus and phosphorylated IκBα levels in the cytoplasm after 15 minutes (Figure 2e + f). In line with their increased activation, SLPI-deficient THP-1 cells had higher levels of phosphorylated NF-κB in the nucleus and increased phosphorylated IκBα levels in the cytoplasm after 15 minutes of LPS stimulation (Figure 2e + f). These data show that endogenous SLPI inhibits NF-κB signaling in monocytic cells in response to microbial signals.

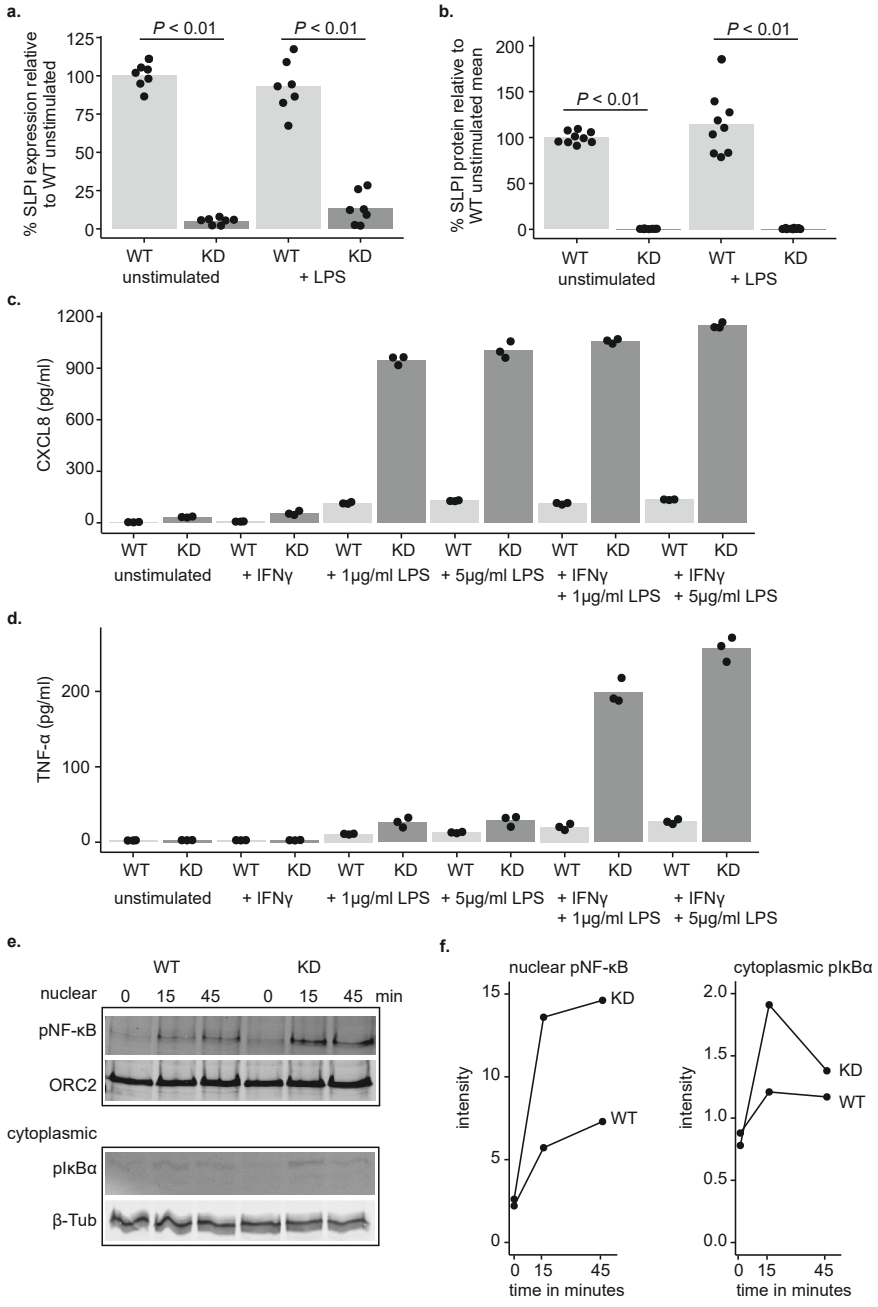


Figure 2: Knockdown of SLPI in THP-1 cells increases CXCL8 and TNF-α production via NF-κB signaling in response to microbial stimulation

THP-1 cells transduced with virus supernatant containing an empty LZRS-IRES-EGFP vector (wildtype, WT) or an empty LZRS-IRES-EGFP vector and shRNA specific for SLPI in a pRSC vector (knockdown, KD) were cultured and stimulated with 1 - 10 µg/mL LPS or left untreated. (a) *SLPI* mRNA expression was measured by qPCR after 24 hours of culture. *SLPI* mRNA expression values relative to *GAPDH* are plotted as percentage of the relative expression in unstimulated WT THP-1. The graph combines data from three experiments and each datapoint represents the mean of two culture wells. Grey bars represent the mean of seven datapoints in each respective group. (b) SLPI protein production in the supernatant was measured by ELISA after 24 hours of culture. SLPI protein abundance is plotted as percentage of the mean SLPI protein in the supernatant of unstimulated WT THP-1 (unstimulated WT THP-1 cells produced SLPI in the range of 281-4390 pg/mL). The graph combines data from four experiments and each datapoint represents a single measurement for one culture well. Grey bars represent the mean of eight or nine datapoints in each respective group. *P*-values were calculated using the Wilcoxon rank sum test (a + b). WT THP-1 cells and KD THP-1 cells were cultured without stimulation or stimulated overnight with 500 U/mL IFN-γ and subsequently cultured with or without 1 µg/mL or 5 µg/mL LPS (c + d + e + f). (c) Supernatant was collected after 3 hours of culture for measurement of CXCL8 protein production by ELISA. (d) Supernatant was collected after 6 hours of culture for measurement of TNF-α protein production by ELISA. The graphs show one representative experiment out of two experiments performed (c + d, see also Supplementary Figures 1a and 1b). Each datapoint represents a single measurement for one culture well (c + d). Grey bars represent the mean of three datapoints in each respective group (c + d). Western blot of phosphorylated NF-κB (pNF-κB) in the nuclear fraction and phosphorylated IκBα (pIκBα) in the cytoplasmic fraction of THP-1 cells transduced with virus supernatant containing an empty LZRS-IRES-EGFP vector (wildtype, WT) or shRNA specific for SLPI in a pRSC vector (knockdown, KD) after stimulation with 7.5 µg/mL LPS for 0, 15 or 45 minutes (e + f). Images of the blots (e) and densitometry of the protein bands relative to loading controls (f) are shown.

To demonstrate that the increased CXCL8 production by SLPI-deficient THP-1 cells is a direct result of lack of SLPI protein, we reconstituted the cells with SLPI. Native SLPI can have two forms: a secreted form with a signal peptide and a truncated form which lacks the signal peptide and is thought to stay intracellularly (18,29). To assess whether both forms of SLPI are able to reconstitute inhibition of CXCL8 production in SLPI-deficient THP-1 cells, we transduced the cells with a retroviral vector containing either truncated or full length SLPI. We detected SLPI protein in the nucleus, the cytoplasm and in the supernatant after reconstitution with either truncated or full length SLPI, suggesting that the signal peptide is not required for transport across the cell membrane of human monocytes (Figure 3a and Supplementary Figure 3a + b). SLPI protein levels were lower after reconstitution with full length SLPI compared to truncated SLPI, which may be due to a lower transduction efficiency of the full length SLPI compared to the truncated SLPI. However, expression of SLPI after reconstitution with full length SLPI was sufficient to suppress LPS-induced CXCL8 production to wildtype levels, confirming that SLPI directly suppresses CXCL8 production in THP-1 cells (Figure 3b and Supplementary Figure 3c). Similarly, reconstitution with truncated SLPI also decreased LPS-induced CXCL8 production to wildtype levels, demonstrating that the signal peptide is not necessary for endogenous SLPI to inhibit chemokine and cytokine production in monocytic cells. In line with the inhibition of CXCL8 production, reconstitution of SLPI-deficient THP-1 cells with either truncated or full length SLPI significantly reduced the amounts of phosphorylated NF-κB in the nucleus at 15 and 45 minutes after LPS stimulation (Figure 3c + d), demonstrating

that SLPI suppresses CXCL8 production via inhibition of NF-κB signaling. The effect of SLPI reconstitution on IκBα phosphorylation was less clear. While, as expected, reconstitution with full length SLPI reduced phosphorylated IκBα in the cytoplasm at 15 minutes after LPS-stimulation, we could not detect reduced IκBα phosphorylation after reconstitution with truncated SLPI in multiple experiments (Figure 3c + d). It is unclear why truncated SLPI has less effect on abundance of phosphorylated IκBα as high concentrations of truncated SLPI are detected in the cytoplasm. A possible explanation is that truncated SLPI, which is abundant in the nuclear fraction, also acts directly in the nucleus by binding to the NF-κB consensus-binding sites within the CXCL8 promoter (9). However, we did not assess this in these experiments. In conclusion, we show that endogenous SLPI expression in monocytic cells suppresses microbiota induced cellular activation. Deficiency of SLPI in THP-1 cells results in increased reactivity to microbial stimulation and reconstitution with either truncated or full length SLPI is able to suppress this activation via inhibition of NF-κB signaling.

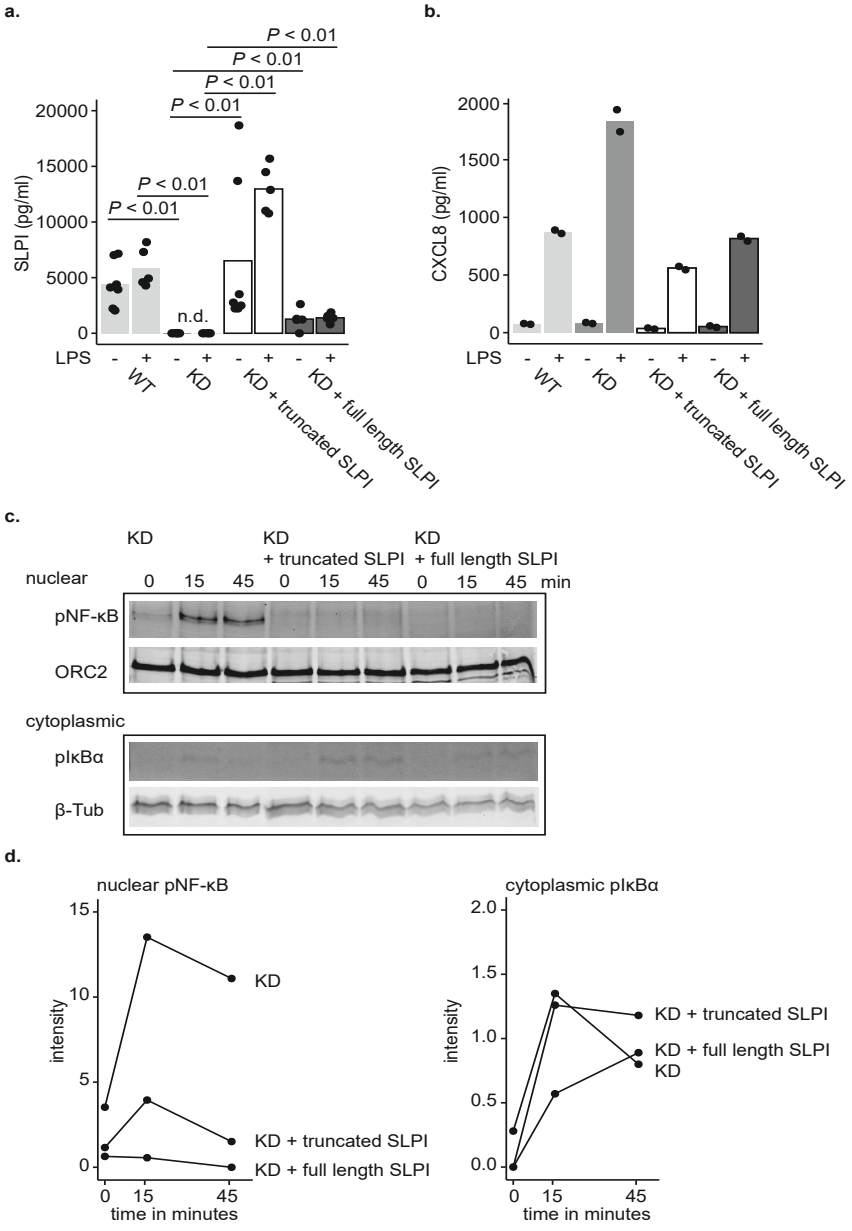


Figure 3: Reconstitution of truncated or full length SLPI in THP-1 cells rescues chemokine inhibition in response to microbial stimulation

THP-1 cells were transduced with virus supernatant containing either an empty LZRS-IRES-EGFP vector (wildtype, WT), or shRNA specific for SLPI in a pRSC vector and an empty LZRS-IRES-EGFP vector (knockdown, KD), or shRNA specific for SLPI in a pRSC vector and truncated SLPI in a LZRS-IRES-EGFP vector (KD + truncated SLPI), or shRNA specific for SLPI in a pRSC vector and full length SLPI in a LZRS-IRES-EGFP vector (KD + full length SLPI). (a) The supernatant was collected after 24 hours of culture with or without 1µg/mL LPS for measurement of SLPI protein production by ELISA. The graph combines data from three experiments and each datapoint represents a single measurement for one culture well. Grey bars represent the mean of five or seven datapoints in each respective group. *P*-values were calculated using the Wilcoxon rank sum test. (b) Supernatant was collected after 24 hours of culture with or without 1µg/mL LPS for measurement of CXCL8 protein production by ELISA. The graph shows one representative experiment out of two experiments performed (b, see also Supplementary Figure 3c). Each datapoint represents a single measurement for one culture well. Grey bars represent the mean of two datapoints in each respective group. Western blot of phosphorylated NF-κB (pNF-κB) in the nuclear fraction and phosphorylated IκBα (pIκBα) in the cytoplasmic fraction of THP-1 cells transduced with virus supernatant containing shRNA specific for SLPI in a pRSC vector and either an empty LZRS-IRES-EGFP vector (knockdown, KD), or a LZRS-IRES-EGFP vector containing truncated SLPI (KD + truncated SLPI), or a LZRS-IRES-EGFP vector containing full length SLPI (KD + full length SLPI) after stimulation with 7.5 µg/mL LPS for 0, 15 or 45 minutes (c + d). Images of the blots (c) and densitometry of the protein bands relative to loading controls are shown (d). n.d. = not detectable.

Exogenous SLPI does not regulate LPS-induced CXCL8 production by THP-1 cells

In the intestine, SLPI is produced in high quantities by epithelial cells (1,2). Previously, it has been postulated that exogenous SLPI uptake would provide a predominant mechanism of monocyte inhibition (8,9,21,36). However, we found that endogenous SLPI is also able to suppresses chemokine and cytokine production in THP-1 cells. This led us to compare the relative contribution of exogenous and endogenous SLPI in inhibition of monocyte activation. Thereto, we cultured SLPI-deficient THP-1 cells in the presence of recombinant human SLPI (without signal peptide) at 10 µg/mL, the concentration also used by others (8,9,21). After culture with the recombinant protein, we detected SLPI in both the nucleus and the cytoplasm of SLPI-deficient THP-1 cells at concentrations comparable to those of endogenous SLPI in wildtype THP-1 cells (Figure 1b and Figure 4a). This demonstrates that recombinant SLPI is taken up by SLPI-deficient THP-1 cells. Next, we examined the effect of exogenous SLPI on LPS-induced CXCL8 production by wildtype THP-1 cells and SLPI-deficient THP-1 cells. CXCL8 release was detectable at 3 hours and 24 hours after LPS-stimulation and was further increased by pretreatment with IFN-γ in both wildtype THP-1 cells and SLPI-deficient THP-1 cells (Figure 4b + c). However, LPS stimulation in the presence of exogenous recombinant SLPI did not inhibit CXCL8 release in wildtype or SLPI knockdown THP-1 cells at either of the timepoints, suggesting that exogenous SLPI is unable to inhibit THP-1 cell activation despite reaching the nucleus and the cytoplasm (Figure 4b + c). To assess whether this also accounted for TNF-α production, SLPI-deficient THP-1 cells were pretreated with IFN-γ and subsequently stimulated with LPS during 6 hours of culture

(Figure 4d). Again, the addition of recombinant SLPI did not inhibit TNF-α production in SLPI-deficient THP-1 cells (Figure 4d). These data argue that endogenous, but not exogenous SLPI regulates LPS-induced activation in THP-1 cells.

Recombinant human SLPI (10 μg/mL) was added to the culture of THP-1 cells transduced with virus supernatant containing shRNA specific for SLPI in a pRSC vector (SLPI knockdown). (a) SLPI protein was measured by ELISA in the nuclear fraction and cytoplasmic fraction of unstimulated SLPI knockdown THP-1 cells after 1 hour of culture. The total protein concentration measured in the nuclear fraction was 2.9 – 3.7 mg/mL and in the cytoplasmic fraction 5.9 – 8.5 mg/mL (data not shown). Wildtype THP-1 cells and THP-1 cells transduced with virus supernatant containing shRNA specific for SLPI in a pRSC vector (SLPI knockdown) were cultured without stimulation, or stimulated with 0.1 μg/mL LPS or stimulated overnight with 500 U/mL IFN-γ and subsequently stimulated with 1 μg/mL LPS (b + c + d). Recombinant human SLPI (10 μg/mL) was added to the culture 1 hour before stimulation with LPS (b + c + d).

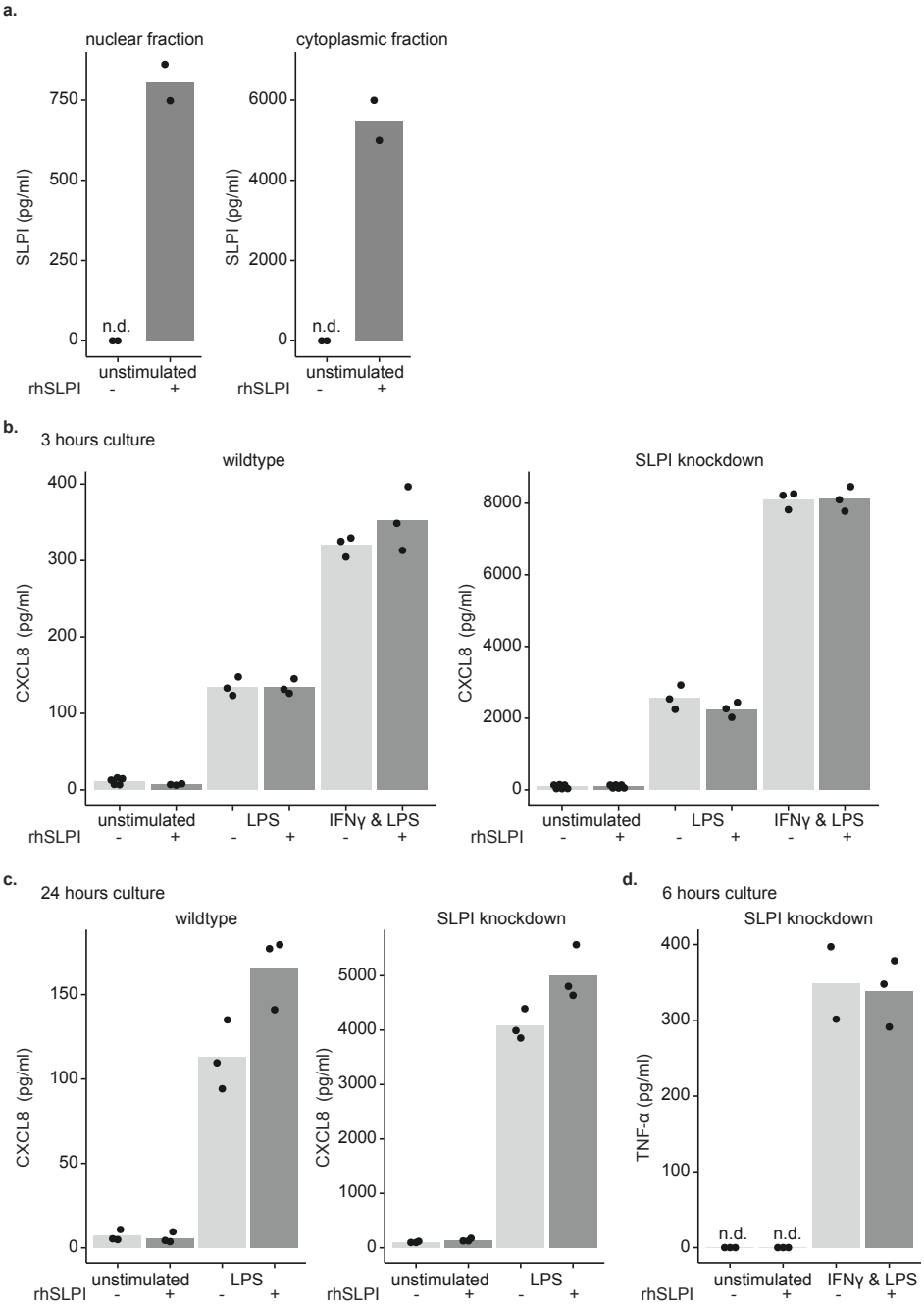


Figure 4: Exogenous SLPI does not regulate LPS-induced CXCL8 production by THP-1 cells

Supernatant was collected after 3 hours of culture (b) or 24 hours of culture (c) for measurement of CXCL8 protein by ELISA. Supernatant was collected after 6 hours of culture for measurement of TNF-α protein by ELISA (d). TNF-α protein was not detectable in the supernatant of wildtype THP-1 cells after 6 hours of culture when unstimulated or stimulated with IFN-γ and LPS, with or without recombinant SLPI (data not shown). The graphs show data from one experiment and each datapoint represents a single measurement for one culture well (a + b + c + d). Grey bars represent the mean of two (a) or three (b + c + d) datapoints in each respective group. n.d. = not detectable.

DISCUSSION

Monocytes need to adapt their threshold of activation to the local tissue environment upon exudation from the blood into the tissue. Here we show that monocytic cells can self-regulate their activation upon microbial stimulation via upregulation of SLPI and that monocytes may thus not be fully dependent on SLPI production by other cell types such as epithelial cells. This implies that in mucosal tissues, contact between micro-organisms and the epithelium may not be required for regulation of monocytes that have recently migrated from the blood. By regulating their own threshold of activation via SLPI, monocytes may be able to adjust to their environment independent of epithelial cell function, thereby preventing tissue damage during inflammation.

We demonstrate that CD68 expressing cells in the human intestinal lamina propria of both IBD-negative patients and patients with Crohn's disease express SLPI. Although this appears to be a small population of SLPI-positive monocytes or macrophages, we detected these cells in the majority of intestinal biopsies from IBD-negative patients (11 out of 15) and Crohn's disease (10 out of 15), demonstrating that SLPI-expressing monocytes or macrophages are present both in health and disease. Whether the number of SLPI producing monocytic cells is different between IBD-negative and Crohn's disease is difficult to quantitate as positive cells in Crohn's disease are often found in small foci, sometimes in areas with crypt loss, while in IBD-negative biopsies the cells appear more evenly dispersed. Hence, the pattern of where SLPI expressing CD68 expressing cells are located might be more relevant to their function in intestinal immune responses.

Endogenous SLPI almost ablates TNF-α and CXCL8 production, as demonstrated by our knock-down and reconstitution experiments, demonstrating that other NF-κB inhibitors including TOLLIP cannot compensate for the loss of SLPI. As the functional analyses were performed with the monocytic cell-line THP-1, we have not yet demonstrated that SLPI inhibits NF-κB-mediated cytokine production in CD68 expressing monocytes/macrophages with equal potency. It was not possible to use

primary monocytes as SLPI expression in peripheral blood monocytes is highly variable from individual to individual and knockdown experiments require high numbers of cells. However, the THP-1 monocytic cell line is a well-known model and the cells have maintained many monocytic functions (37). Therefore, our data suggest that endogenous SLPI inhibits microbial-induced activation of human monocytes *in vivo*.

Our finding that exogenous SLPI does not regulate LPS-induced monocyte activation is in contrast to the previously reported inhibitory effect of recombinant human SLPI in the human myelomonocytic cell line U937 (9). It can be questioned whether the differential effect of recombinant SLPI is due to a different mechanism of uptake in THP-1 cells compared to U937 cells. SLPI has been shown to bind to the phospholipid scramblase 1 (PLSCR1) and 4 (PLSCR4), membrane proteins that regulate the movement of phospholipids between the inner and the outer plasma membrane (38). In addition, PLSCR1 modulates phagocytosis and can be detected in fully internalized phagosomes (38,39). However, mRNA expression of PLSCR1 is approximately equal in unstimulated THP-1 cells and U937 cells and PLSCR4 is absent in both cell-lines (source: the Human Protein Atlas project (33,34)). Therefore, differences in PLSCR1 expression are not expected to explain the discrepancy between our findings and those of others. However, it remains possible that localization and intracellular trafficking of endogenous SLPI is different from that of exogenous SLPI, particularly as we observe that endogenous SLPI, but not exogenous SLPI, is able to inhibit NF-κB signaling in THP-1 cells.

It is unknown which factors induce SLPI expression in monocytes. In human epithelial cells, SLPI expression is upregulated by a range of TLR ligands and cytokines including TNF-α and IL-1β (2,16,26). In murine macrophages, SLPI expression is induced by LPS and suppressed by IFN-γ (18). However, in our hands SLPI expression in freshly isolated peripheral blood monocytes could not be upregulated by direct incubation with a range of TLR ligands even after pre-incubation with IFN-γ (data not shown). Possibly, extravasation into the tissue, encounter of local tissue cytokines and environmental factors stimulate SLPI expression in monocytes in a manner specifically tailored to residency in each tissue. As SLPI inhibits LPS-induced chemokine and cytokine production by monocytes, we speculate that regulation of monocyte activation by SLPI is especially important in tissues where tight control of the microbiota-host interaction is crucial for homeostasis, such as the intestines and the lungs.

ACKNOWLEDGEMENTS & FUNDING

We thank Lissy de Ridder for collecting intestinal biopsies and resection material of pediatric Crohn's disease patients and controls. We thank Willem Korstiaan Smits and Beatriz Calado for help with analysis and preparing the figures.

Sandrine Nugteren was funded by the Dutch Digestive Foundation (grant registration number: Focus Project 15-17); Janneke N. Samsom received the grant.

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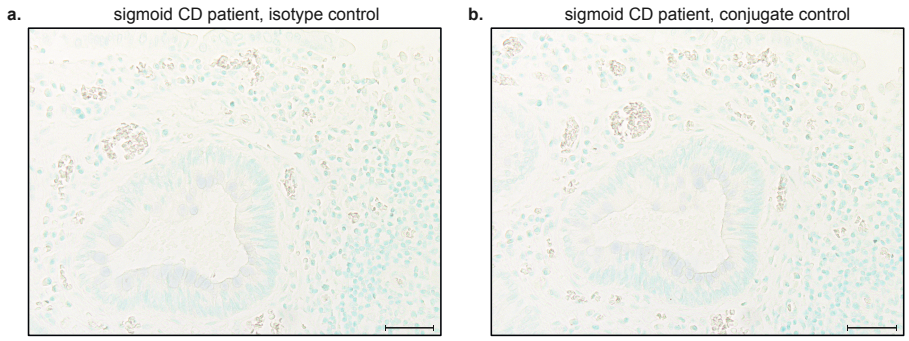
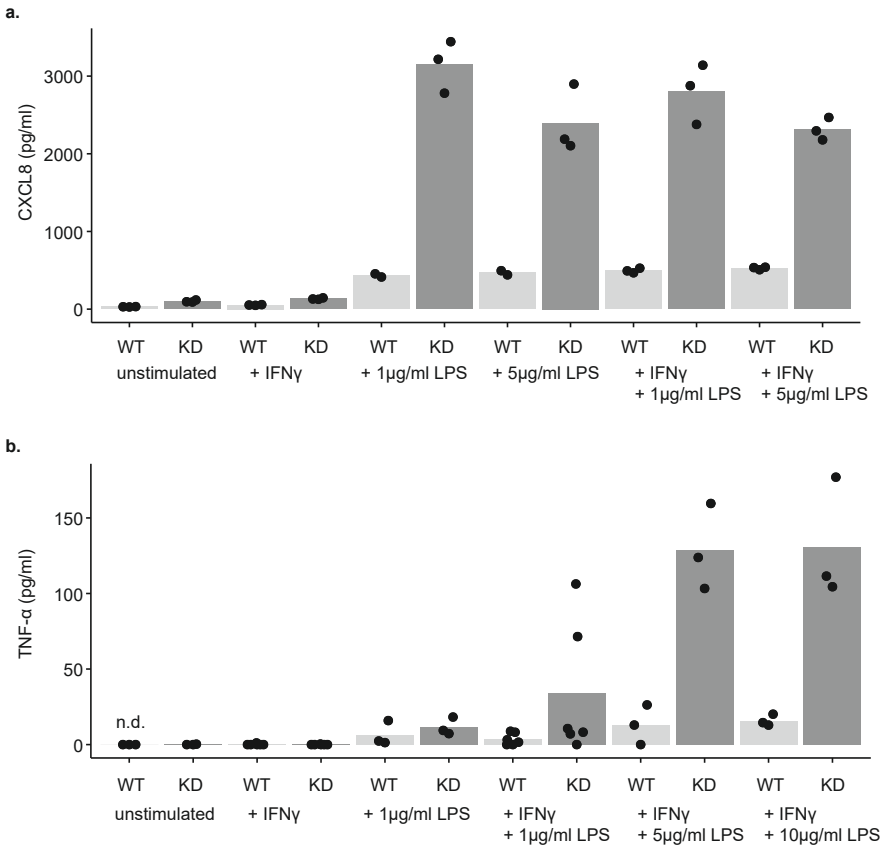


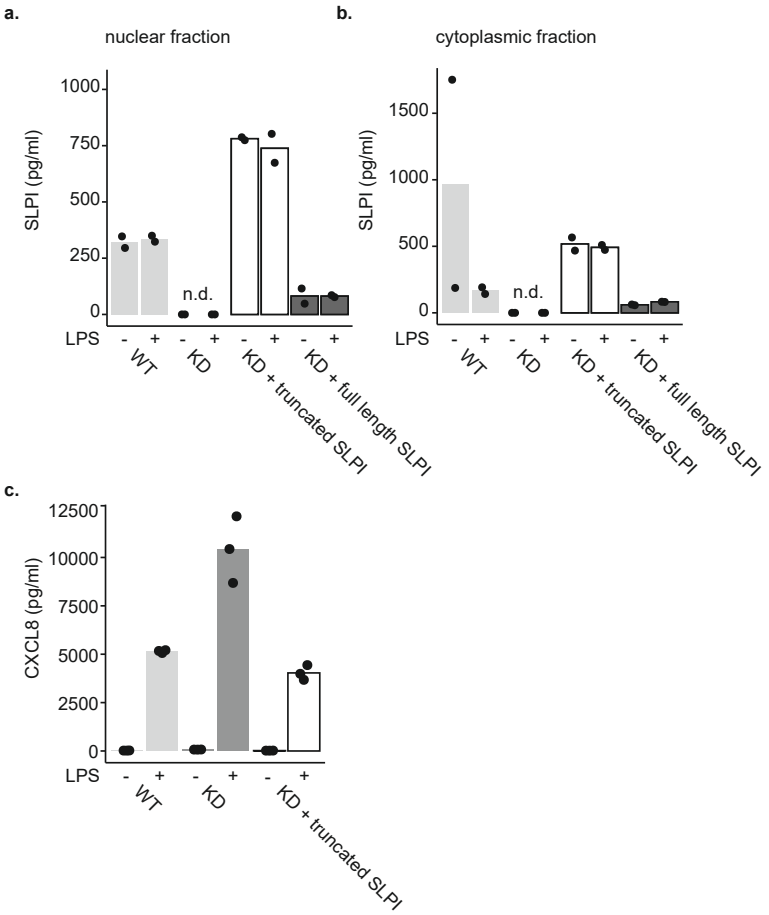
Supplementary figure 1: Isotype control and conjugate control for SLPI and CD68 immunohistochemistry

(a) Immunohistochemistry was performed according to the protocol for the double staining for SLPI and CD68 protein expression with isotypes (goat IgG for SLPI and mouse IgG1 for CD68) instead of primary antibodies on resection material from a pediatric Crohn's disease (CD) patient. (b) In addition, as a conjugate control, immunohistochemistry was performed according to the protocol for the double staining for SLPI and CD68 protein without addition of a primary antibody or isotype on a serial section from the same paraffin block as in panel a. Scale bars represent 100 µm.



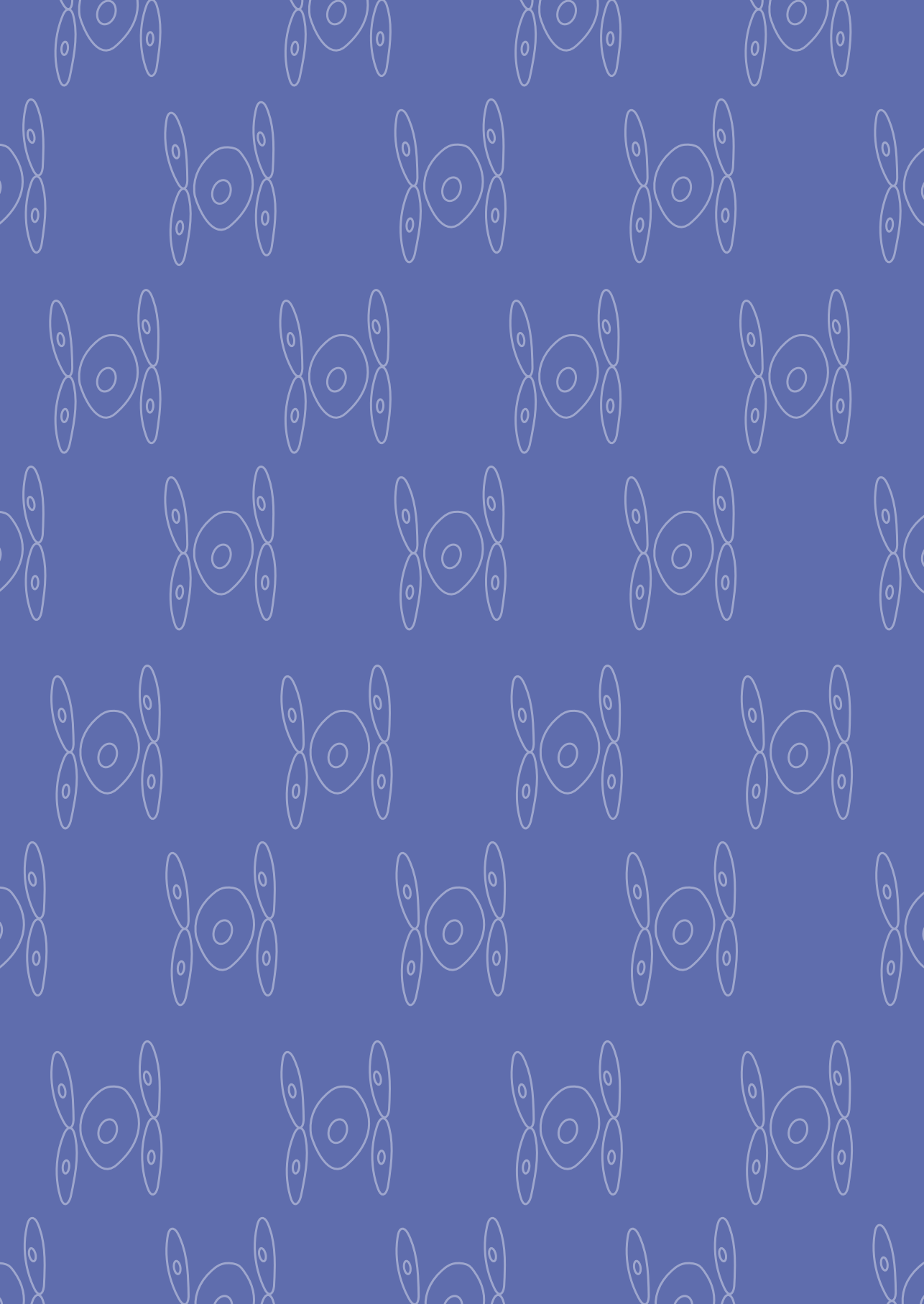
Supplementary figure 2: Knockdown of SLPI in THP-1 cells increases CXCL8 and TNF-α production via NF-κB signaling in response to microbial stimulation

Wildtype THP-1 cells (wildtype, WT) and THP-1 cells transduced with virus supernatant containing shRNA specific for SLPI in a pRSC vector (knockdown, KD) were cultured without stimulation or stimulated overnight with 500 U/mL IFN-γ and subsequently cultured with or without 1 µg/mL, 5 µg/mL or 10 µg/mL LPS. (a) Supernatant was collected after 3 hours of culture for measurement of CXCL8 protein production by ELISA. Grey bars represent the mean of two or three datapoints in each respective group. (b) Supernatant was collected after 6 hours of culture for measurement of TNF-α protein production by ELISA. Grey bars represent the mean of three or six datapoints in each respective group. The graphs show data from one experiment and each datapoint represents a single measurement for one culture well (a + b). n.d. = not detectable.



Supplementary figure 3: Reconstitution of truncated SLPI in THP-1 cells rescues chemokine inhibition in response to microbial stimulation

THP-1 cells were transduced with virus supernatant containing either an empty LZRS-IRES-EGFP vector (wildtype, WT), or shRNA specific for SLPI in a pRSC vector and an empty LZRS-IRES-EGFP vector (knockdown, KD), or shRNA specific for SLPI in a pRSC vector and truncated SLPI in a LZRS-IRES-EGFP vector (KD + truncated SLPI), or shRNA specific for SLPI in a pRSC vector and full length SLPI in a LZRS-IRES-EGFP vector (KD + full length SLPI). The nuclear fraction (a) and cytoplasmic fraction (b) were collected after 24 hours of culture with or without 1µg/mL LPS for measurement of SLPI protein production by ELISA. The graphs show data from one experiment and each datapoint represents a single measurement for one culture well (a + b). Grey bars represent the mean of two datapoints in each respective group. (c) Supernatant was collected after 24 hours of culture with or without 1µg/mL LPS for measurement of CXCL8 protein production by ELISA. The graph shows data from one experiment and each datapoint represents a single measurement for one culture well. Grey bars represent the mean of three datapoints in each respective group. n.d. = not detectable.



CHAPTER 5

Expression of the immune modulator secretory leukocyte protease inhibitor (SLPI) in colorectal cancer liver metastases and matched primary tumors is associated with a poorer prognosis

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ABSTRACT

Secretory leukocyte protease inhibitor (SLPI), a pleiotropic protein expressed by healthy intestinal epithelial cells, functions as an inhibitor of NF-κB and neutrophil proteases and exerts antimicrobial activity. We previously showed SLPI suppresses intestinal epithelial chemokine production in response to microbial contact. Increased SLPI expression was recently detected in various types of carcinoma. In addition, accumulating evidence indicates SLPI expression is favorable for tumor cells. In view of these findings and the abundance of SLPI in the colonic epithelium, we hypothesized SLPI promotes colorectal cancer (CRC) growth and metastasis. Here, we aimed to establish whether SLPI expression in CRC is related to clinical outcome. Using a cohort of 507 patients with CRC who underwent resection of liver metastases, we show that high SLPI protein expression in both liver metastases and primary CRC is associated with significantly shorter overall survival after resection of liver metastases. The prognostic value of SLPI in CRC patients with liver metastases implies a role for SLPI in the formation of metastasis of human CRC. Based on the immune regulatory functions of SLPI, we anticipate that expression of SLPI provides tumors with a mechanism to evade infiltration by immune cells.

INTRODUCTION

The pleiotropic protein secretory leukocyte protease inhibitor (SLPI) is constitutively expressed and secreted by human epithelial cells (1,2). SLPI exerts diverse functions, including the ability to act as a potent NF-κB inhibitor (3) and inhibit proteases such as neutrophil elastase (4) and also exhibits broad antimicrobial properties (5-7). We previously showed that repetitive microbial contact induced expression of SLPI in intestinal epithelial cells and that SLPI suppressed chemokine production in response to microbial signals by inhibiting NF-κB activation (8). Thus, SLPI prevents excessive inflammation during intestinal homeostasis.

Tumors frequently modulate the expression of immunomodulatory proteins to evade anti-tumor immune responses. Most investigations of immune invasion in CRC have focused on the interactions between tumor cells and T cells or natural killer (NK) cells (9,10). However, innate immune proteins can also regulate the anti-tumor immune response (11). Increased SLPI protein expression is observed in several types of cancer, including colorectal cancer (CRC) (12), gastric cancer (13), non-small cell lung cancer (14) and ovarian cancer (15). While the roles of SLPI in tumor formation and progression have not been fully elucidated, multiple human and mouse studies indicate a role for SLPI in the formation of metastases. In particular, in a mouse model of polyclonal breast cancer, clones expressing SLPI entered the vasculature and formed metastases more efficiently than clones that did not express SLPI (16). In addition, SLPI promoted spontaneous lung metastasis in an orthotopic mouse model of breast cancer (17). Moreover, high tumor *SLPI* mRNA expression was associated with shorter overall survival in patients with triple negative breast cancer (17) and expression of SLPI was associated with poorer five-year overall survival in gastric cancer (13). However, some studies have not reported a tumor promoting role for SLPI. For example, overexpression of SLPI in lung carcinoma cells reduced the number of liver metastases in a mouse model (18). This protective effect was associated with suppressed production of TNF-α and E-selectin in the liver, suggesting that formation of liver metastases in this model requires a proinflammatory environment (18). Thus, the precise role of SLPI in cancer has not yet been fully elucidated.

The role of SLPI in human CRC has not been investigated. However, SLPI was one of four secreted proteins upregulated in the conditioned medium of a highly metastatic human colorectal cancer cell line compared to the poorly metastatic parental cell line (17). In addition, overexpression of SLPI enhanced tumor growth in murine colon cancer cells (19). In view of the immunoregulatory functions of SLPI in the intestine and its potential tumor-promoting role, we hypothesized that SLPI promotes tumor

growth and metastasis in CRC. In this study, we aimed to establish whether expression of SLPI in human CRC metastases is associated with patient survival.

The liver is the most common site of metastasis in CRC; 25-30% of patients with CRC develop colorectal cancer liver metastases (CRCLM) (20-22). Approximately 25% of patients with CRCLM are eligible for surgical resection of the affected part of the liver, which is currently the only treatment with curative intent for CRC patients with liver metastases (23,24). However, survival outcomes after resection of CRCLM are highly variable, even among patients with similar clinical and pathology-based risk scores (25). A better understanding of tumor biology may help to predict survival for patients with CRCLM. Using a series of 507 patients with CRC who underwent resection of liver metastases, we show that SLPI protein expression in CRCLM and the matched primary tumors is associated with shorter overall survival.

MATERIALS AND METHODS

Patient cohort and tissue microarray (TMA) generation

Histologically confirmed, formaldehyde-fixed paraffin-embedded (FFPE) CRCLM tissue samples and, when available, samples of the corresponding primary tumor were collected from 507 patients who underwent resection of CRCLM between 1990 and 2010 in seven Dutch hospitals (the DeCoDe PET group), as described previously (26). A previous power calculation indicated a sample size of 361 patients was required for a similar analysis (26). We assumed similar proportions of patients would exhibit low and high SLPI expression (50:50); therefore, we assumed that the sample size of this cohort (n = 507) would be sufficient. Patients with more than one primary tumor were excluded from this study. Tissue microarrays (TMAs) were generated from the original FFPE tissue blocks, according to previously described protocols (27). From every paraffin block, three tissue core biopsies of 0.6 millimeter in diameter were punched from morphologically representative areas and transferred into recipient TMA paraffin blocks. Four-micrometer TMA sections were cut and subsequently mounted onto glass slides. Collection, storage and use of the tissue samples and clinical data were conducted in compliance with the Dutch code of conduct for responsible use of human tissue for medical research (28).

SLPI immunohistochemistry

TMA sections were deparaffinized in xylene and rehydrated in alcohol, incubated in 3% H_2O_2 in PBS for 20 min to quench endogenous peroxidase activity, and antigen retrieval was performed by microwave treatment in citrate buffer (10 mM, pH 6.0). Sections were blocked for one hour at room temperature in Tris buffer (10 mM, pH

8.0) containing 5 mM EDTA (pH 8.0), 0.15 M NaCl, 0.25% gelatin, 0.05% Tween-20 and 10% normal human serum (AB serum; Sanquin, Amsterdam, The Netherlands) plus 10% normal horse serum (Biowest, Nuaillé, France) or 10% normal rabbit serum (Jackson ImmunoResearch, West Grove, PA, USA) matching the species in which the secondary antibody was raised. Subsequently, the sections were stained with either a monoclonal anti-human-SLPI antibody (4 µg/mL, mouse IgG1, HM2037, clone 31; HycultBiotech, Uden, The Netherlands) or polyclonal anti-human-SLPI antibody (1 µg/mL, goat IgG, BAF1274; R&D Systems/Bio-Techne, Minneapolis, MN, USA) in PBS overnight at 4 °C. Immunoreactive sites were detected by incubation with a biotinylated horse-anti-mouse antibody (1:500, Vector Laboratories, Burlingame, CA, USA) or biotinylated rabbit-anti-goat antibody (1:500, Vector Laboratories) for one hour at room temperature. Biotinylated antibodies were detected using a complex of avidin and biotin (Vectastain ABC Elite Kit, Vector Laboratories) and 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, Zwijndrecht, The Netherlands). Sections were counterstained with hematoxylin (Vector Laboratories) and subsequently dehydrated and immersed in xylene.

Scoring of SLPI expression

Images of stained sections were digitally captured using an Aperio AT2 scanner (Leica Microsystems B.V., Amsterdam, The Netherlands) equipped with a 20× / 0.75 objective (UPlanSAPO; Olympus, Leiderdorp, The Netherlands). The intensity of SLPI protein expression in the cytoplasm of neoplastic epithelial cells was manually scored in a semi-quantitative manner as 'negative', 'weak', 'moderate' or 'strong' using the online platform Slide Score (www.slidescore.com). The scoring strategy was designed in consultation with a pathologist and based on the range of SLPI staining intensity observed in tumor cells; all sections were scored by the same investigator. In order to assess the reproducibility of the scoring, a second pathologist independently scored > 20% of the cores stained with the monoclonal antibody and > 20% of the cores stained with the polyclonal antibody, based on images of the scoring categories shown in Figure 1a+b and Figure 3a+b. Both observers were blinded to the clinicopathological information at time of assessment. The linear weighted kappa values were for 0.62 for the monoclonal antibody and 0.63 for the polyclonal antibody, indicating fair to good interobserver agreement. The scoring method was agreed on by both observers and discrepancies were discussed by the observers.

Statistical analysis

For each tissue type (CRCLM or primary tumor) and anti-SLPI antibody, the maximum score from the one to three TMA cores stained for each patient was used for analysis. Patients for whom none of the three cores were evaluable were excluded from the

respective analyses (Supplementary figure 1). Overall survival was defined as the time after resection of CRCLM until death in months, with a maximum follow-up period of 60 months. Patients who died within 2 months of CRCLM resection were excluded from the analysis, to avoid bias related to death due to surgical complications (Supplementary figure 1). Patients with missing survival status or follow-up data were also excluded from the analysis (Supplementary figure 1).

The prognostic value of SLPI protein expression in the liver metastases and primary tumors was evaluated separately by 500-fold cross-validation (26). In short, in each of the 500 cycles, the study population was randomly divided into equally sized training and validation sets. The optimal cut-off for dichotomizing the training set was calculated in every cycle using receiver operating characteristic (ROC) curve analysis for 3-year overall survival. This cut-off was subsequently applied to the validation set to calculate a cross-validated hazard rate ratio (HRR) for 3-year overall survival in univariable Cox regression analysis. In addition, a corrected HRR was calculated in each validation cycle by multivariable Cox regression analysis, which included the following established clinical prognostic factors: number of CRCLM > 1, primary tumor-to-CRCLM interval < 12 months, lymph node positivity at time of CRC diagnosis, and maximal CRCLM diameter > 5.0 cm (29). In both the univariable and multivariable analysis, the average HRR (HRR_{av}) of the 500 HRRs was used and the *P*-value was calculated based on the percentage of HRR < 1 over the 500 cycles. The relation between SLPI expression and overall survival was visualized by Kaplan Meier curves both before and after dichotomization. Dichotomization for each antibody was performed using the cut-off that was most frequently selected during the automated 500-fold cross-validation procedure. Patients were classified as 'SLPI-low' or 'SLPI-high' based on the cross-validated cut-offs for each antibody. The log-rank test was used to determine whether overall survival varied significantly between the two groups. In addition, the time points at which 50% of the patients had died (median overall survival) and 95% confidence intervals were calculated for both groups. The clinicopathological features of the SLPI-high and SLPI-low groups were compared using Pearson's Chi-squared test for categorical variables and the Kruskal-Wallis rank sum test for non-normally distributed continuous variables. The relationship between SLPI detected using the monoclonal antibody and SLPI detected using the polyclonal antibody was examined using Fisher's exact test (two-sided). The relationship between SLPI expression in liver metastases and the corresponding primary tumors was also tested using Fisher's exact test (two-sided). All statistical analyses and visualization were performed using R version 3.5.1 (30). The 'survival' (31,32), 'survminer' (33), 'pROC' (34) and 'survivalROC' (35) packages were employed for survival analysis and cross-validation. The data in

this study is reported in compliance with the REMARK recommendations for reporting tumor marker prognostic studies (36).

RESULTS

SLPI is expressed in a subset of CRCLM

We assessed SLPI protein expression in CRCLM tissue samples from a Dutch cohort of 507 patients to establish the prognostic value of SLPI in CRCLM. The characteristics of this study population have been described previously (26). To robustly assess the prognostic value of SLPI, we detected SLPI protein expression by immunohistochemistry using two different antibodies: a monoclonal antibody raised against human SLPI purified from sputum and a polyclonal antibody raised against *Escherichia coli*-derived recombinant human SLPI. CRCLM tissue samples stained with the SLPI monoclonal antibody from 386 patients were available for analysis and CRCLM samples stained with the SLPI polyclonal antibody from 372 patients were available for analysis (Supplementary figure 1a).

In whole tissue sections of primary CRC samples from 10 patients, SLPI expression was homogeneous within each section, but varied between tumors from different patients (data not shown). SLPI expression was mainly observed in the cytoplasm on the luminal side of the tumor cells (Figure 1a + b). We detected expression of SLPI in CRCLM in 45% of patients using the monoclonal antibody and in CRCLM in 72% of patients using the polyclonal antibody (Figure 1c + d). Overall, SLPI protein expression was detected in the CRCLM samples of a substantial subgroup of patients.

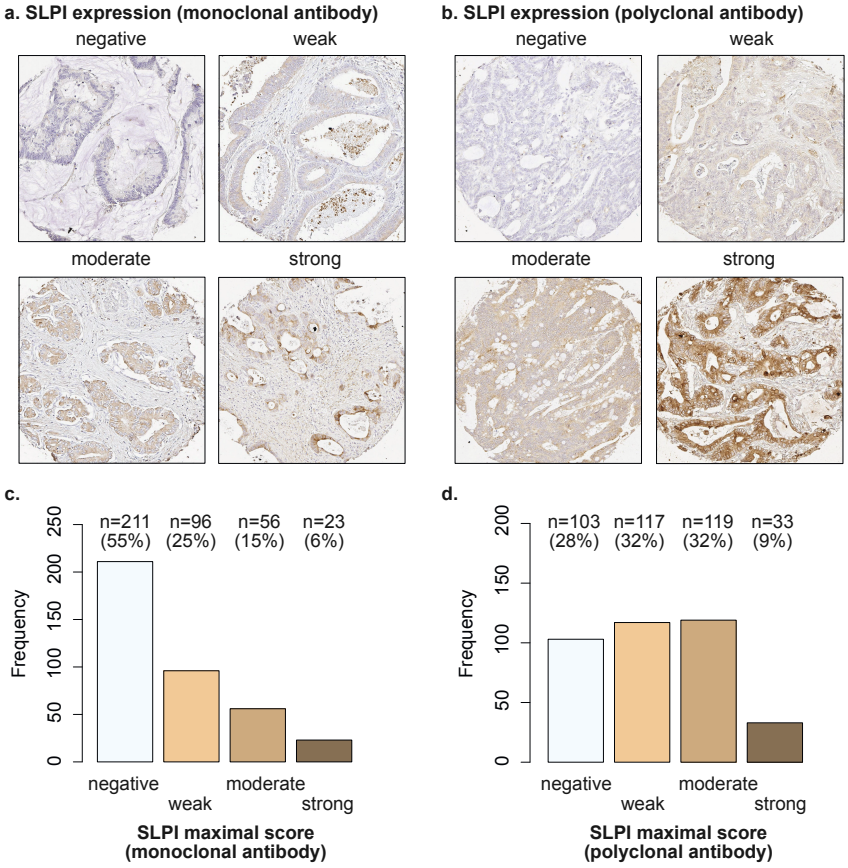


Figure 1: SLPI is expressed in a subset of CRCLM

Examples of TMA cores of CRCLM stained for SLPI using the monoclonal antibody (a) or polyclonal antibody (b); SLPI staining intensity in tumor cells was scored. The frequencies and percentages of CRCLM scored as 'negative', 'weak', 'moderate' or 'strong' after staining with the monoclonal SLPI antibody (c) or polyclonal SLPI antibody (d) are shown; only the maximal score for each patient was included.

Expression of SLPI in CRCLM is associated with shorter overall survival

In order to assess the prognostic value of SLPI expression in CRCLM, we determined the optimal cut-offs for dichotomization of the cohort using 500-fold cross-validation.

For CRCLM stained with the SLPI monoclonal antibody, the optimal cut-off in all 500 cross-validation cycles was negative vs. weak/moderate/strong SLPI expression (data not shown). The patients were classified as 'SLPI-low' or 'SLPI-high' based on this cross-validated cut-off. Using this cut-off, high SLPI expression in CRCLM was associated with significantly shorter overall survival compared to low SLPI expression, with an average

hazard rate ratio (HRR_{av}) of 1.43 ($P = 0.02$; Supplementary figure 2a). Furthermore, patients with high SLPI expression in CRCLM had significantly shorter overall survival after CRCLM resection compared to patients with low SLPI expression (log-rank test: $P = 0.04$; Figure 2a+b). The median overall survival time for patients with high SLPI expression was 44 months (95% confidence interval: 38-60 months) compared to over 60 months (lower limit of the 95% confidence interval: 52 months) for patients with low SLPI expression in CRCLM (Figure 2b).

Using the polyclonal antibody, the optimal dichotomization cut-off was negative/weak (low) vs. moderate/strong (high) SLPI expression in all 500 cross-validation cycles (data not shown). Patients with high SLPI expression tended to have shorter overall survival compared to patients with low SLPI expression, though the HRR_{av} of 1.33 was not statistically significant ($P = 0.07$; Supplementary figure 2c). In the survival analysis, overall survival was not significantly different between the SLPI-high and SLPI-low groups for CRCLM stained using the polyclonal antibody (log-rank test: $P = 0.10$, Figure 2c + d). Median overall survival time was 41 months for the SLPI-high group (lower limit of the 95% confidence interval: 34 months, upper limit more than 60 months) and 58 months (lower limit of the 95% confidence interval: 48 months, upper limit more than 60 months) for the SLPI-low group (Figure 2d).

Patient age, gender, the location of the primary tumor, the grade of differentiation of the primary tumor, the size of the primary tumor, presence of lymph node metastases, presence of extrahepatic metastases, the interval between the primary tumor diagnosis and detection of liver metastases, the size of the liver metastases and the number of liver metastases were not significantly different between patients with high or low SLPI expression in CRCLM tissues stained with either the SLPI monoclonal antibody or the polyclonal antibody (Supplementary figure 2a + b).

We also compared the SLPI expression scores for CRCLM from 357 patients stained with both the monoclonal and polyclonal antibody (Supplementary figure 4a). There was a significant association between detection of high SLPI expression with the monoclonal antibody and detection of high SLPI expression with the polyclonal antibody (two-sided Fisher's exact test: $P < 0.01$). For 74% of patients, both the monoclonal and polyclonal antibody resulted in the same classification, either 'SLPI-low' or 'SLPI-high' (Supplementary figure 4a). SLPI expression was more frequently scored as 'weak' in CRCLM stained with the polyclonal antibody when no staining was detected using the monoclonal antibody than vice-versa, indicating the monoclonal antibody has a higher threshold of detection for SLPI. In conclusion, patients with high SLPI expression in CRCLM, as detected using the monoclonal antibody, have significantly shorter overall survival compared to patients with low SLPI expression in CRCLM.

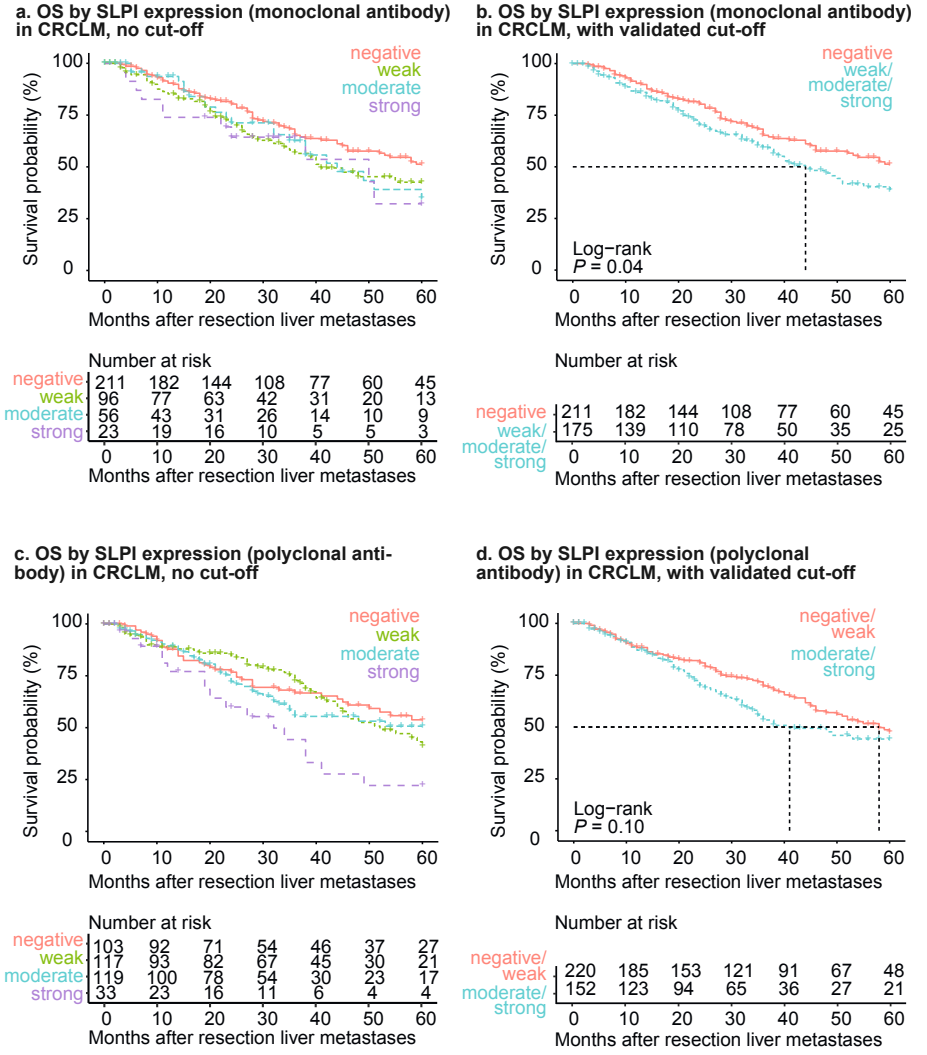


Figure 2: SLPI expression in CRCLM is associated with shorter overall survival

Kaplan-Meier overall survival curves after resection of liver metastases (in months) for the CRCLM study population. Overall survival was stratified by SLPI expression after staining with the monoclonal antibody (a + b) or polyclonal antibody (c + d). Curves without a cut-off (a + c) and with the cut-off calculated from the 500-fold cross-validation procedure (b + d) are shown. *P*-values were calculated using the log-rank test. The dotted lines represent the time point at which 50% of the group had died (median overall survival time). OS = overall survival.

SLPI expression in CRCLM has prognostic value independently of established clinical risk factors

Next, we determined whether expression of SLPI in CRCLM has prognostic value independently of established clinical risk factors. The following factors have been demonstrated to be associated with shorter overall survival after resection of liver metastases in patients with CRCLM: more than one CRCLM, a primary tumor-to-CRCLM interval less than 12 months, lymph node positivity at time of CRC diagnosis, a maximal CRCLM diameter > 5.0 cm and a serum carcinoembryonic antigen (CEA) level > 200 ng/mL (29). The prognostic value of these parameters was previously extensively assessed in the current cohort (26). The presence of more than one liver tumor was significantly associated with shorter overall survival. A primary tumor-to-CRCLM interval less than 12 months, lymph node positivity at the time of CRC diagnosis and a maximal CRCLM diameter > 5.0 cm were also associated with shorter overall survival (HRR > 1), though these trends were not statistically significant (26). Serum CEA > 200 ng/mL was not associated with overall survival in this cohort (26). Therefore, we only included more than one CRCLM, a primary tumor-to-CRCLM interval less than 12 months, lymph node positivity at the time of CRC diagnosis, and a maximal CRCLM diameter > 5.0 cm in the multivariable Cox regression model.

Importantly, the prognostic value of SLPI expression detected using the monoclonal antibody was not confounded by these established clinical risk factors; high SLPI expression had a HRR_{av} of 1.63 for overall survival in the multivariable model ($P = 0.02$; Supplementary figure 2b). Detection of high SLPI expression using the polyclonal antibody was also associated with shorter overall survival compared to low SLPI expression in the multivariable model, though this trend was not statistically significant (HRR_{av} 1.37; $P = 0.10$; Supplementary figure 2d). Thus, detection of SLPI expression in CRCLM using the monoclonal antibody was associated with significantly shorter overall survival after surgical resection of liver metastases, independently of other known clinical risk factors.

SLPI is expressed in a subset of primary tumors from patients with CRCLM

It is currently unknown whether SLPI expression alters during the progression of tumors to metastasis. Therefore, we compared SLPI expression in CRCLM samples and primary CRC tissues; 168 paired CRCLM and the matched primary tumors from the same patients were available for this analysis (Supplementary figure 1b). We observed similar patterns of SLPI expression in the primary tumor samples and CRCLM samples, with SLPI mainly expressed in the cytoplasm on the luminal side of the tumor cells (Figure 3a + b). SLPI expression was detected in the primary tumors of 60% of patients

using the monoclonal antibody and 87% of patients using the polyclonal antibody (Figure 3c + d).

Using the monoclonal antibody to detect SLPI expression in primary CRC, the optimal cut-off for dichotomizing the patients was negative (low) vs. weak/moderate/strong (high) in 263 of the 500 cycles (data not shown). Based on this cut-off, the monoclonal antibody detected high SLPI expression in both the primary tumor and CRCLM in 29% of patients and detected low SLPI expression in both the primary tumor and CRCLM in 26% of patients (Supplementary figure 5a). Expression of SLPI in the primary tumor was significantly associated with expression of SLPI in the corresponding liver metastases (two-sided Fisher's exact test: $P < 0.01$). Using the monoclonal antibody, a higher proportion of primary tumors exhibited high SLPI expression than the matched CRCLM (Supplementary figure 5a).

Using the polyclonal antibody to detect SLPI expression in primary CRC, the optimal cut-off for dichotomization was negative/weak (low) vs. moderate/strong (high) in 480 out of 500 cycles (data not shown). In 28% of patients, both the primary tumor and CRCLM expressed low levels of SLPI (Supplementary figure 5b). We detected high SLPI expression in both the primary tumor and CRCLM in 37% of patients. As observed for the monoclonal antibody, high SLPI expression in primary CRC was significantly associated with high SLPI expression in CRCLM using the polyclonal antibody (two-sided Fisher's exact test: $P = 0.02$). Moreover, using the polyclonal antibody, more primary tumors exhibited high SLPI expression than the matched CRCLM (Supplementary figure 5b).

Overall, these results indicate SLPI expression in primary tumor samples is related to SLPI expression in CRCLM samples, which leads to the question of whether expression of SLPI in primary CRC is associated with the prognosis of patients with CRCLM.

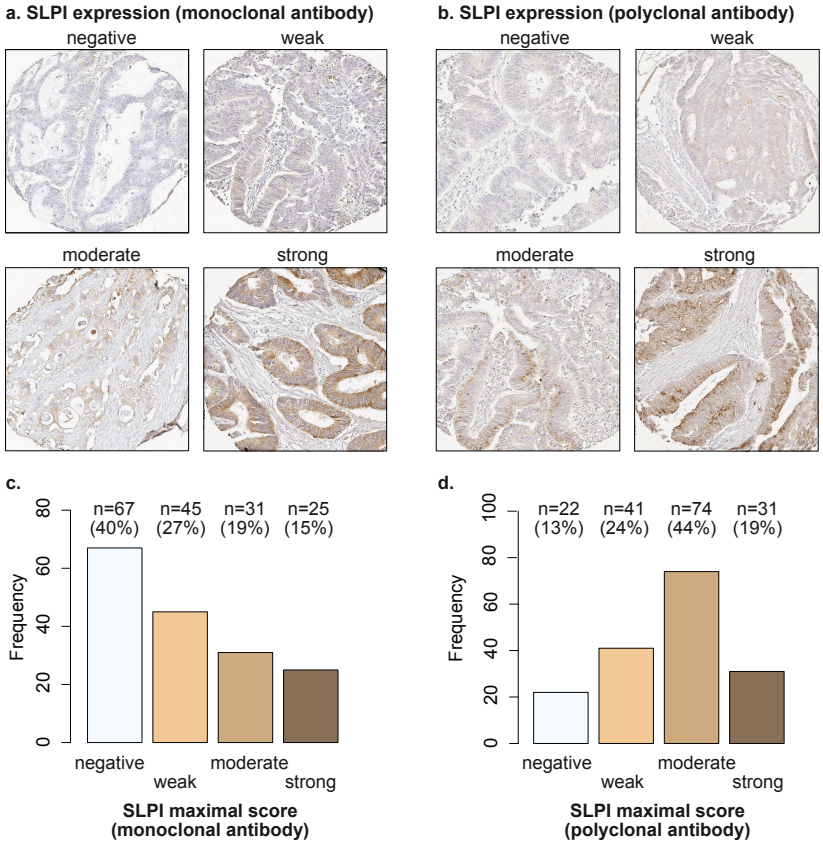


Figure 3: SLPI is expressed in a subset of primary tumors from patients with CRCLM

Examples of TMA cores of primary CRC tumors stained for SLPI using the monoclonal antibody (a) or polyclonal antibody (b); SLPI staining intensity in tumor cells was scored. Frequencies and percentages of primary tumors scored as 'negative', 'weak', 'moderate' or 'strong' after staining with the SLPI monoclonal antibody (c) or SLPI polyclonal antibody (d) are shown; only the maximal score for each patient was included.

SLPI expression in primary CRC is associated with shorter overall survival in patients with CRCLM

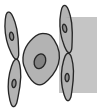
In order to establish whether primary tumor SLPI expression has prognostic value in patients with CRCLM undergoing surgical resection of their metastases, we examined the association between SLPI expression in primary CRC and overall survival after CRCLM resection.

Detection of high SLPI expression in the primary tumor using the monoclonal antibody was associated with significantly shorter overall survival after CRCLM resection compared to low SLPI expression, with a HRR_{av} of 1.80 ($P = 0.02$, Supplementary figure 6a). Furthermore, in the survival analysis, patients with high SLPI expression in the primary tumor had significantly shorter overall survival after CRCLM resection compared to patients with low SLPI expression in the primary tumor (log-rank test: $P = 0.03$; Figure 4a + b). The median overall survival time of patients with high SLPI expression in the primary tumor was 46 months after CRCLM resection (lower limit of 95% confidence interval: 32 months; upper limit, > 60 months), compared to > 60 months (lower limit of 95% confidence interval: 52 months) for patients with low SLPI expression (Figure 4b).

Detection of high SLPI expression in primary tumors using the polyclonal antibody was also associated with poorer overall survival, with a HRR_{av} of 1.25; however, this effect was not statistically significant ($P = 0.24$; Supplementary figure 6c). In addition, in the survival analysis there was no significant difference in overall survival between patients with low and high SLPI expression in the primary tumor detected using the polyclonal antibody (log-rank test: $P = 0.44$; Figure 4c + d). Moreover, the median overall survival time for patients with high SLPI expression was 52 months (lower limit of the 95% confidence interval: 41 months, upper limit > 60 months), compared to > 60 months (lower limit of the 95% confidence interval, > 60 months) in patients with low SLPI expression based on the polyclonal antibody (Figure 4d).

Expression of SLPI detected in primary CRC using either the monoclonal or polyclonal antibody was not related to patient age, the grade of differentiation of the primary tumor, primary tumor size, presence of lymph node metastases, presence of extrahepatic metastases, the interval between primary tumor diagnosis and detection of liver metastases, or the size or number of liver metastases (Supplementary figure 3c + d). However, for primary tumors stained using the monoclonal antibody, the SLPI-high group more frequently had a primary tumor on the left side of the colon compared to the SLPI-low group (Pearson's Chi-squared test: $P = 0.04$; Supplementary figure 3c). However, we did not observe this association in analysis of primary tumors stained using the polyclonal antibody (Supplementary figure 3d). In addition, in analysis of primary tumors stained with the polyclonal antibody, more patients in the SLPI-high group were female than in the SLPI-low group (Pearson's Chi-squared test: $P = 0.03$; Supplementary figure 3d), though we did not observe a similar association using the monoclonal antibody.

We were able to compare the SLPI scores for primary tumors stained using the monoclonal and polyclonal antibody for 157 patients (Supplementary figure 4b). There was a significant association between detection of SLPI with the monoclonal antibody and the polyclonal antibody (two-sided Fisher's exact test, $P < 0.01$). Moreover, the monoclonal and polyclonal antibodies resulted in the same classification as either 'SLPI-low' or 'SLPI-high' in 72% of patients, (Supplementary figure 4b). As observed for the CRCLM, we observed relatively lower SLPI staining scores using the monoclonal antibody than the polyclonal antibody in primary tumors.



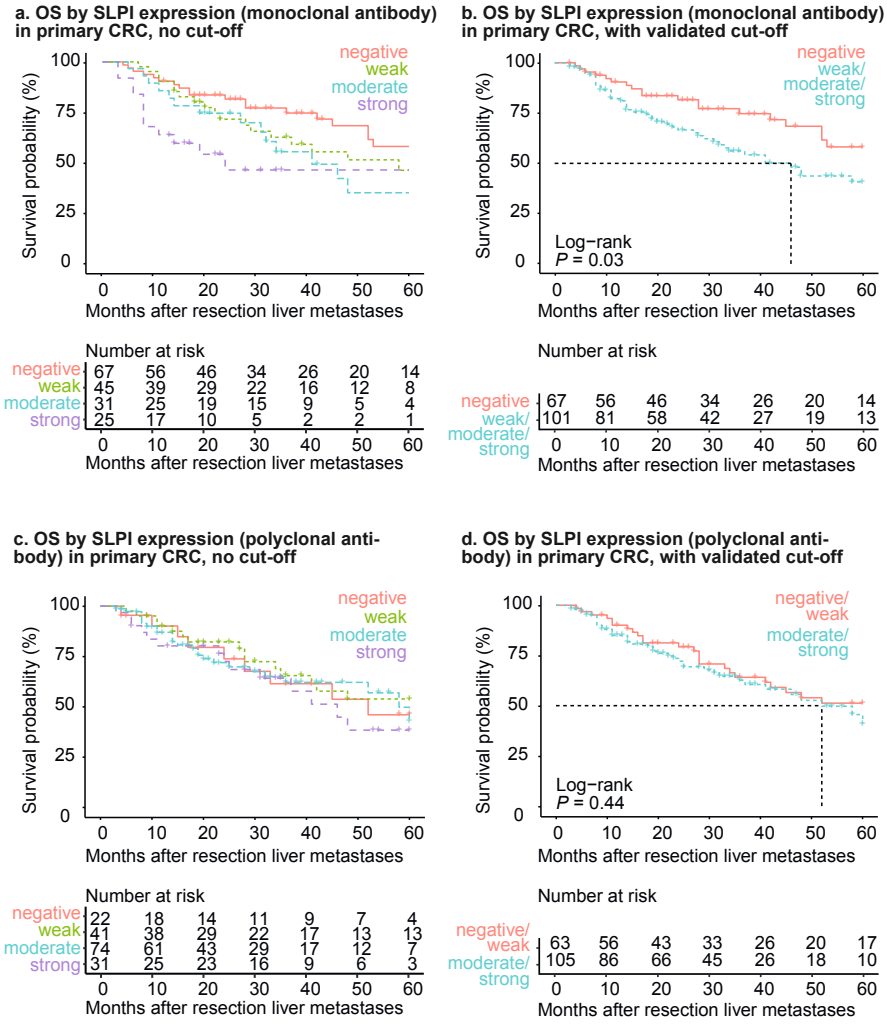


Figure 4: SLPI expression in primary CRC is associated with shorter overall survival in patients with CRCLM

Kaplan-Meier overall survival curves after resection of liver metastases (in months) for the CRCLM study population. Overall survival was stratified by SLPI expression after staining with the monoclonal antibody (a + b) or polyclonal antibody (c +d). Curves without a cut-off (a + c) and with the cut-off calculated from the 500-fold cross-validation procedure (b + d) are shown. *P*-values were calculated using the log-rank test. The dotted lines represent the time point at which 50% of the group had died (median overall survival time). OS = overall survival.

In conclusion, detection of high SLPI expression in the primary tumors using the monoclonal antibody was associated with shorter overall survival after surgical resection of liver metastases in patients with CRCLM.

SLPI expression in primary CRC has prognostic value in patients with CRCLM independently of established clinical risk factors

Next, we investigated whether SLPI expression in primary CRC had independent prognostic value in patients with CRCLM by adjusting for previously established clinical risk factors. Using the multivariable model described earlier, detection of high SLPI expression in the primary tumor using the monoclonal antibody was associated with significantly shorter overall survival after resection of liver metastases compared to low SLPI expression (HRR_{av} 1.86, $P = 0.04$; Supplementary figure 6b). For primary tumors stained with the polyclonal antibody, high SLPI expression was not significantly associated with shorter overall survival in the multivariable model (HRR_{av} 1.26; P=0.28; Supplementary figure 6d).

In conclusion, detection of high SLPI expression in primary tumor samples with the monoclonal antibody was significantly associated with shorter overall survival, independently of established clinical prognostic factors.

DISCUSSION

SLPI is a small protein produced in large quantities by healthy epithelial cells throughout the body. The many functions of SLPI include modulation of the immune response via suppression of chemokine production (8). Recent studies indicated that SLPI drives metastasis in mammary carcinoma (16,17), but the role of SLPI in CRC tumor formation and progression is poorly characterized. In this analysis of a large cohort of patients with CRC who underwent resection of liver metastases, we demonstrate high expression of SLPI in both the liver metastases and primary tumors is associated with shorter overall survival. The prognostic value of SLPI was independent of established clinical risk factors that were previously associated with poorer overall survival in this cohort (26). Therefore, our findings indicate that assessment of SLPI expression could help to predict the prognosis of patients with CRC after resection of liver metastases. Subsequent mechanistic analyses are required to investigate whether SLPI plays a causal role in CRC and may reveal previously unknown mechanisms involved in metastasis.

In the healthy intestine, SLPI prevents tissue damage by inhibiting neutrophil proteases and exerting antimicrobial activity and suppresses infiltration of immune cells to maintain intestinal homeostasis (8,37). Therefore, in CRC, SLPI may predominantly act on the tumor microenvironment, rather than on tumor cell proliferation itself. Most proteins known to mediate immune evasion in CRC, including programmed death ligand 1 (PD-L1) and CEA, act by suppressing T cell or NK cell activation in

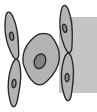
the tumor niche (9,10). Although the exact functional role of SLPI in CRC remains to be determined, it is likely that the pleiotropic functions of SLPI promote immune evasion via multiple processes. Firstly, as it directly suppresses the production of chemokines by intestinal epithelial cells under homeostasis, SLPI may also directly suppress chemokine gradients and thus prevent recruitment of immune cells to the tumor niche. This effect may be clinically relevant, as the absence of tumor infiltrating lymphocytes in both primary CRC (38) and CRCLM (39) is associated with shorter overall survival. Secondly, soluble SLPI can exert generalized immune suppression by acting as an NF-κB inhibitor (40,41). Lastly, the function of SLPI as a protease inhibitor may provide tumor cells with the capacity to defend their niche by inhibiting the protease activity of infiltrating immune cells (4). Moreover, multiple functions of SLPI may promote tumor metastasis. In particular, SLPI enhanced the formation of vessel-like structures and increased the metastatic potential of tumor cells in a mouse model of polyclonal mammary carcinoma (16). The anticoagulative activity of SLPI partly explained the ability of SLPI to drive metastasis in this model (16). Given its diverse activities, SLPI is likely to exert a number of functions in various processes related to tumor growth and metastasis. Future studies are required to elucidate the precise role of SLPI in processes related to the progression and metastasis of CRC, including immune modulation.

Other proteins with significant prognostic value in this cohort are aurora kinase A (AURKA), epidermal growth factor receptor (EGFR), prostaglandin-endoperoxide synthase 2 (PTGS2, also known as cyclooxygenase-2), glucose transporter 1 (SLC2A1) and vascular endothelial growth factor A (VEGFA) (26,42,43). This study demonstrates SLPI expression has similar prognostic value in patients with CRCLM as these proteins. However, SLPI is likely to have a different biological function in CRC compared to these other prognostic proteins. In short, both AURKA and EGFR promote sustained proliferation of tumor cells, SLC2A1 expression is related to anaerobic glycolysis, VEGFA promotes angiogenesis and contributes to induction of regulatory T cells (44) and PTGS2 is involved in both the proliferation and invasion of tumor cells. In addition, PTGS2-derived prostaglandin E_2 acts on the tumor niche as it promotes activation of myeloid-derived suppressor cells, and thereby inhibits cytotoxic T cell and NK cell activation (45). We hypothesize that SLPI prevents the recruitment of immune cells to the tumor, in contrast to VEGFA and PTGS2 which suppress the activation of T cells or NK cells in the tumor niche.

SLPI protein expression in both the primary CRC tissues and liver metastases varied substantially between patients. However, SLPI was invariably localized to the cytoplasm, mainly on the luminal side of the tumor cells, which is similar to the

expression pattern observed in healthy intestinal epithelial cells (37). The factors that trigger SLPI expression in some tumors, but not in others, remain unclear. We observed SLPI was strongly expressed in a subgroup of primary CRC tumors, and in these cases, high SLPI expression in the primary tumor was positively associated with high SLPI expression in the matched CRCLM, which indicates that SLPI may already be upregulated in non-metastatic tumor cells. Interestingly, SLPI was expressed at relatively higher levels in primary tumors than in the corresponding liver metastases, indicating that SLPI may be downregulated during the progression to metastasis, possibly due to the influence of the local environment in the liver. High SLPI expression in primary CRC was also associated with shorter overall survival after resection of the liver metastases, suggesting that assessment of SLPI expression in primary CRC samples holds prognostic value for patients for whom resected liver metastases are not available.

We used two antibodies to detect SLPI expression, one monoclonal and one polyclonal. We observed the same result using both antibodies: high SLPI expression in CRCLM and high SLPI expression in primary tumors were associated with shorter overall survival after resection of liver metastases in patients with CRCLM. Overall, the monoclonal antibody led to lower SLPI expression scores than the polyclonal antibody, which could be explained by the fact that monoclonal antibodies only recognize one epitope, in contrast to polyclonal antibodies. Indeed, we found that the monoclonal antibody was better at discriminating patients with a poorer prognosis, as the association between SLPI expression and overall survival was only significant for the monoclonal antibody. However, the similar results obtained using the monoclonal and polyclonal antibody strengthen our argument that SLPI protein expression is related to prognosis in CRCLM patients.



In conclusion, high SLPI expression in both CRCLM and primary CRC are associated with a poorer prognosis after resection of liver metastases. Further elucidation of the involvement of SLPI in various tumor-promoting processes may help to identify new targets for cancer therapy. In view of the role of SLPI in intestinal homeostasis, we suggest that SLPI influences the anti-tumor immune response in CRC.

ACKNOWLEDGEMENTS

We thank the DeCoDe PET group for collecting the patient tissue samples, Jan Hudeček for developing and making Slide Score available to us, Menno de Vries for managing the clinical database, Veerle M.H. Coupé for helping to write the script for the 500-fold

cross-validation procedure and advice on statistical analyses, and Dimitris Rizopoulos for advice on the statistical analyses.

FUNDING

This study was funded by the Dutch Digestive Foundation (grant registration number: Focus Project 15-17).

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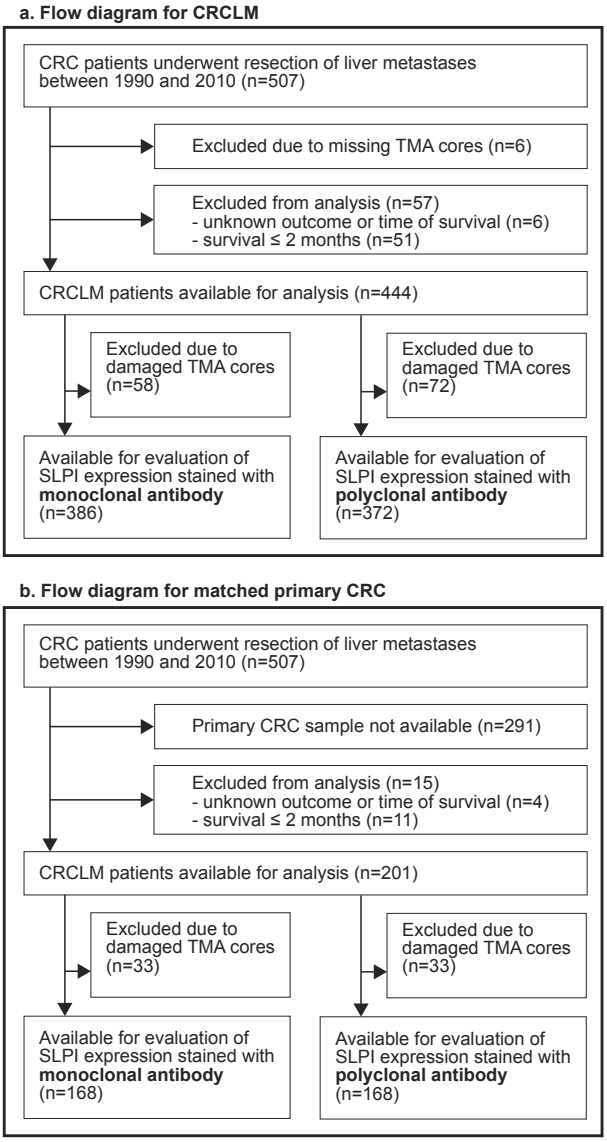
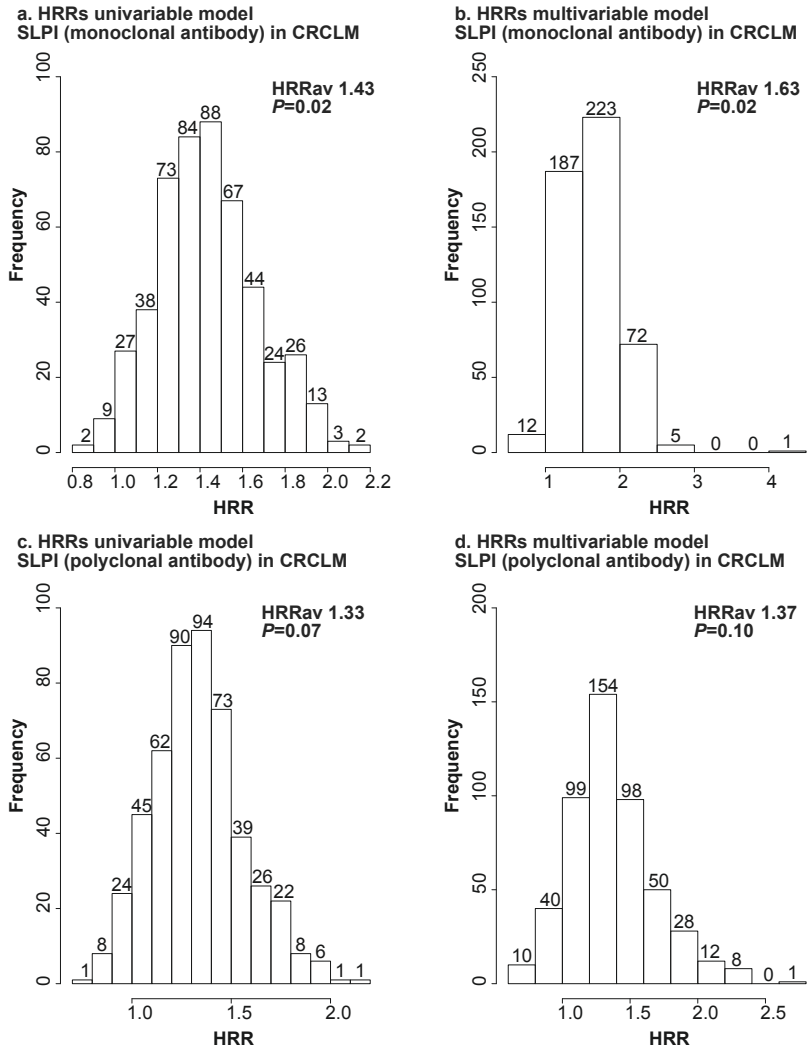


Supplementary figure 1: Flow diagrams of the study cohort

In total, 507 patients with CRC underwent resection of CRCLM in Dutch hospitals between 1990 and 2010. Patients with overall survival of 2 months or less, unknown outcome or missing follow-up data were excluded. After excluding patients for whom none of the three cores could be scored due to technical reasons, SLPI expression was scored in CRCLM stained with the monoclonal antibody from 386 patients (a), CRCLM stained with the polyclonal antibody from 372 patients (a), matched primary CRC tissues stained with the monoclonal antibody from 168 patients (b), and matched primary CRC tissues stained with the polyclonal antibody in 168 patients (b).



Supplementary figure 2: Distribution of cross-validated HRRs for SLPI expression in CRCLM

The optimal cut-off for SLPI expression in CRCLM was calculated and cross-validated using 3-year overall survival after resection of liver metastases as the outcome. The distribution of HRRs is shown for SLPI scores after staining with the monoclonal antibody (a + b) and polyclonal antibody (c + d). Both univariable analyses (a + c) and multivariable analyses (b + d) are shown (see methods). HRRav = average hazard rate ratio over all 500 cycles.

a. Characteristics of patients included in the analysis of SLPI expression in CRCLM using the monoclonal antibody

Clinicopathological variable	**SLPI-low (n=211)**	**SLPI-high (n=175)**	**Total (n=386)**	***P*-value**
Age in years, mean (SD)	61.4 (9.6)	62.9 (10.7)	62.1 (10.1)	0.08[1]
Gender female male	76 (36%) 135 (64%)	61 (35%) 114 (65%)	137 (36%) 249 (65%)	0.81[2]
Primary tumor location right (cecum until flexura lienalis) left (flexura lienalis to rectum)	32 (16%) 172 (84%)	39 (22%) 134 (78%)	71 (19%) 306 (81%)	0.09[2]
Primary tumor histological grade well-differentiated moderately differentiated moderately-poorly differentiated poorly differentiated	7 (5%) 115 (82%) 7 (5%) 12 (9%)	5 (4%) 111 (86%) 5 (4%) 8 (6%)	12 (4%) 226 (84%) 12 (4%) 20 (7%)	0.80[2]
Primary tumor maximal diameter in mm, mean (SD)	45.1 (19.6)	43.6 (18.8)	44.4 (19.2)	0.65[1]
Positive lymph nodes detected yes no	73 (53%) 65 (47%)	75 (58%) 55 (42%)	148 (55%) 120 (45%)	0.43[2]
Extrahepatic metastases yes no	14 (7%) 176 (93%)	11 (7%) 152 (93%)	25 (7%) 328 (93%)	0.82[2]
Time between primary tumor and liver metastases in months, mean (SD)	18.7 (17.3)	19.5 (20.8)	19.0 (19.0)	0.78[1]
Maximal diameter of liver metastases, mean (SD)	43.1 (28.2)	40.1 (21.3)	41.8 (25.3)	0.68[1]
Number of liver metastases, mean (SD)	2.0 (1.6)	2.0 (1.3)	2.0 (1.5)	0.40[1]

[1]Kruskal-Wallis rank sum test

[2]Pearson's Chi-squared test

b. Characteristics of patients included in the analysis of SLPI expression in CRCLM using the polyclonal antibody

Clinicopathological variable	SLPI-low (n=339)	SLPI-high (n=152)	Total (n=372)	*P*-value
Age in years, mean (SD)	61.8 (9.8)	63.1 (10.3)	62.3 (10.0)	0.15[1]
Gender female male	73 (33%) 147 (67%)	55 (36%) 97 (64%)	128 (34%) 244 (66%)	0.55[2]
Primary tumor location right (cecum until flexura lienalis) left (flexura lienalis to rectum)	40 (19%) 174 (81%)	30 (20%) 119 (80%)	70 (19%) 293 (81%)	0.73[2]
Primary tumor histological grade well-differentiated moderately differentiated moderately-poorly differentiated poorly differentiated	7 (5%) 120 (83%) 7 (5%) 11 (8%)	6 (5%) 96 (85%) 5 (4%) 6 (5%)	13 (5%) 216 (84%) 12 (5%) 17 (7%)	0.90[2]
Primary tumor maximal diameter in mm, mean (SD)	46.6 (19.9)	43.3 (18.7)	45.0 (19.4)	0.33[1]
Positive lymph nodes detected yes no	80 (56%) 63 (44%)	64 (56%) 50 (44%)	144 (56%) 113 (44%)	0.98[2]
Extrahepatic metastases yes no	11 (6%) 188 (95%)	12 (9%) 126 (91%)	23 (7%) 314 (93%)	0.26[2]
Time between primary tumor and liver metastases in months, mean (SD)	18.6 (18.6)	18.6 (18.6)	18.6 (18.6)	0.90[1]
Maximal diameter of liver metastases, mean (SD)	42.6 (27.1)	40.7 (23.2)	41.9 (25.6)	0.63[1]
Number of liver metastases, mean (SD)	2.1 (1.6)	2.0 (1.4)	2.0 (1.5)	0.69[1]

[1]Kruskal-Wallis rank sum test
[2]Pearson's Chi-squared test

c. Characteristics of patients included in the analysis of SLPI expression in primary CRC tissues using the monoclonal antibody

Clinicopathological variable	**SLPI-low (n=67)**	**SLPI-high (n=101)**	**Total (n=168)**	***P*-value**
Age in years, mean (SD)	63.1 (10.2)	62.5 (10.8)	62.7 (10.5)	0.80[1]
Gender female male	20 (30%) 47 (70%)	37 (37%) 64 (63%)	57 (34%) 111 (66%)	0.36[2]
Primary tumor location right (cecum until flexura lienalis) left (flexura lienalis to rectum)	20 (30%) 47 (70%)	16 (16%) 83 (84%)	36 (22%) 130 (78%)	0.04[2]
Primary tumor histological grade well-differentiated moderately differentiated moderately-poorly differentiated poorly differentiated	3 (5%) 51 (86%) 0 (0%) 5 (9%)	4 (4%) 77 (82%) 6 (6%) 7 (7%)	7 (5) 128 (84%) 6 (4%) 12 (8%)	0.27[2]
Primary tumor maximal diameter in mm, mean (SD)	47.8 (19.3)	44.2 (19.4)	45.6 (19.4)	0.22[1]
Positive lymph nodes detected yes no	36 (57%) 27 (43%)	58 (59%) 41 (41%)	94 (58%) 68 (42%)	0.86[2]
Extrahepatic metastases yes no	5 (8%) 58 (92%)	12 (12%) 85 (88%)	17 (11%) 143 (89%)	0.37[2]
Time between primary tumor and liver metastases in months, mean (SD)	19.1 (22.1)	16.3 (16.3)	17.4 (18.8)	0.84[1]
Maximal diameter of liver metastases, mean (SD)	40.8 (24.1)	39.0 (20.8)	39.7 (22.1)	0.75[1]
Number of liver metastases, mean (SD)	1.9 (1.4)	2.0 (1.2)	1.9 (1.3)	0.20[1]

[1]Kruskal-Wallis rank sum test
[2]Pearson's Chi-squared test

d. Characteristics of patients included in the analysis of SLPI expression in primary CRC tissues using the polyclonal antibody

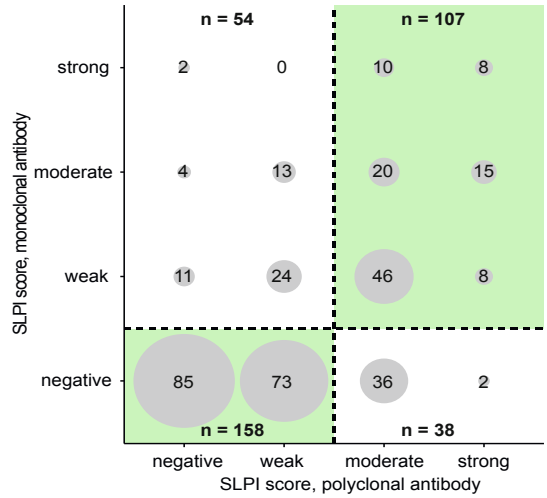
Clinicopathological variable	SLPI-low (n=63)	SLPI-high (n=105)	Total (n=168)	*P*-value
Age in years, mean (SD)	64.4 (8.4)	63.4 (11.4)	63.8 (10.4)	0.98[1]
Gender female male	16 (25%) 47 (75%)	44 (42%) 61 (58%)	60 (36%) 108 (64%)	0.03[2]
Primary tumor location right (cecum until flexura lienalis) left (flexura lienalis to rectum)	16 (26%) 46 (74%)	21 (20%) 83 (80%)	37 (22%) 129 (78%)	0.40[2]
Primary tumor histological grade well-differentiated moderately differentiated moderately-poorly differentiated poorly differentiated	1 (2%) 46 (84%) 2 (4%) 1 (2%)	8 (8%) 82 (84%) 3 (3%) 5 (5%)	9 (6%) 128 (84%) 5 (3%) 11 (7%)	0.25[2]
Primary tumor maximal diameter in mm, mean (SD)	49.1 (21.2)	42.5 (18.5)	45.0 (19.8)	0.07[1]
Positive lymph nodes detected yes no	31 (53%) 28 (48%)	61 (59%) 42 (41%)	92 (57%) 70 (43%)	0.41[2]
Extrahepatic metastases yes no	6 (10%) 55 (90%)	10 (10%) 87 (90%)	16 (10%) 142 (90%)	0.92[2]
Time between primary tumor and liver metastases in months, mean (SD)	18.8 (22.6)	17.8 (17.4)	18.2 (19.5)	0.71[1]
Maximal diameter of liver metastases, mean (SD)	42.2 (24.8)	37.6 (21%)	39.2 (22.5)	0.33[1]
Number of liver metastases, mean (SD)	1.9 (1.3)	1.9 (1.3)	1.9 (1.3)	0.57[1]

[1]Kruskal-Wallis rank sum test
[2]Pearson's Chi-squared test

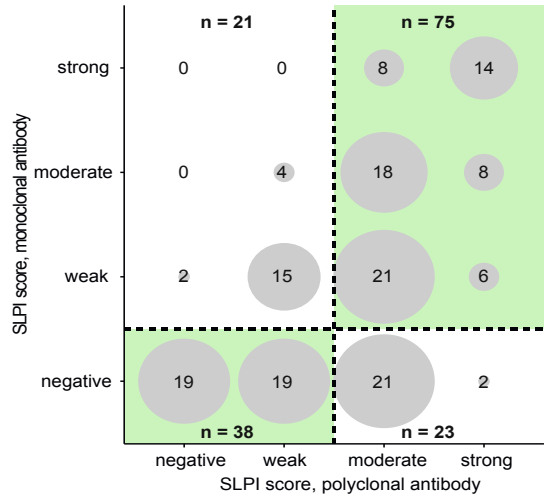
Supplementary figure 3: Clinicopathological characteristics of patients included in the analysis of SLPI expression in either CRCLM or primary CRC

Characteristics of the patients included in the analysis of SLPI expression in CRCLM tissues (a + b) or primary CRC tissues (c + d) stained with the monoclonal antibody (a+c) or polyclonal antibody (b + d). Patients were classified as 'SLPI-low' or 'SLPI-high' based on the cross-validated cut-offs.

a. Relation between monoclonal and polyclonal SLPI antibody staining scores in CRCLM

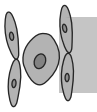


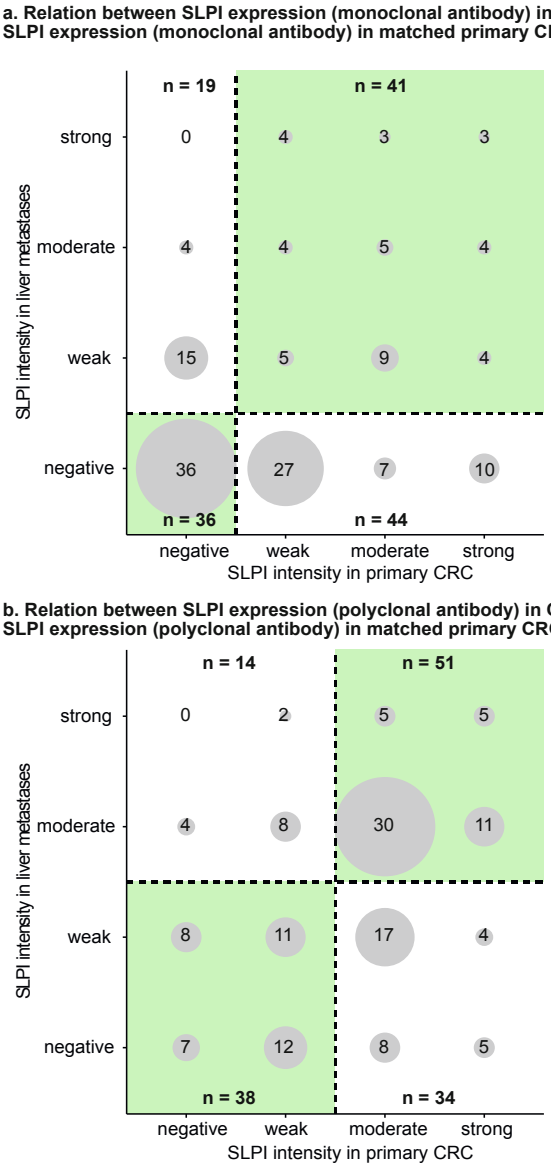
b. Relation between monoclonal and polyclonal SLPI antibody staining scores in primary CRC



Supplementary figure 4: Relationship between the SLPI scores of CRCLM stained with the monoclonal and polyclonal antibody, and between primary tumors stained with the monoclonal and polyclonal antibody.

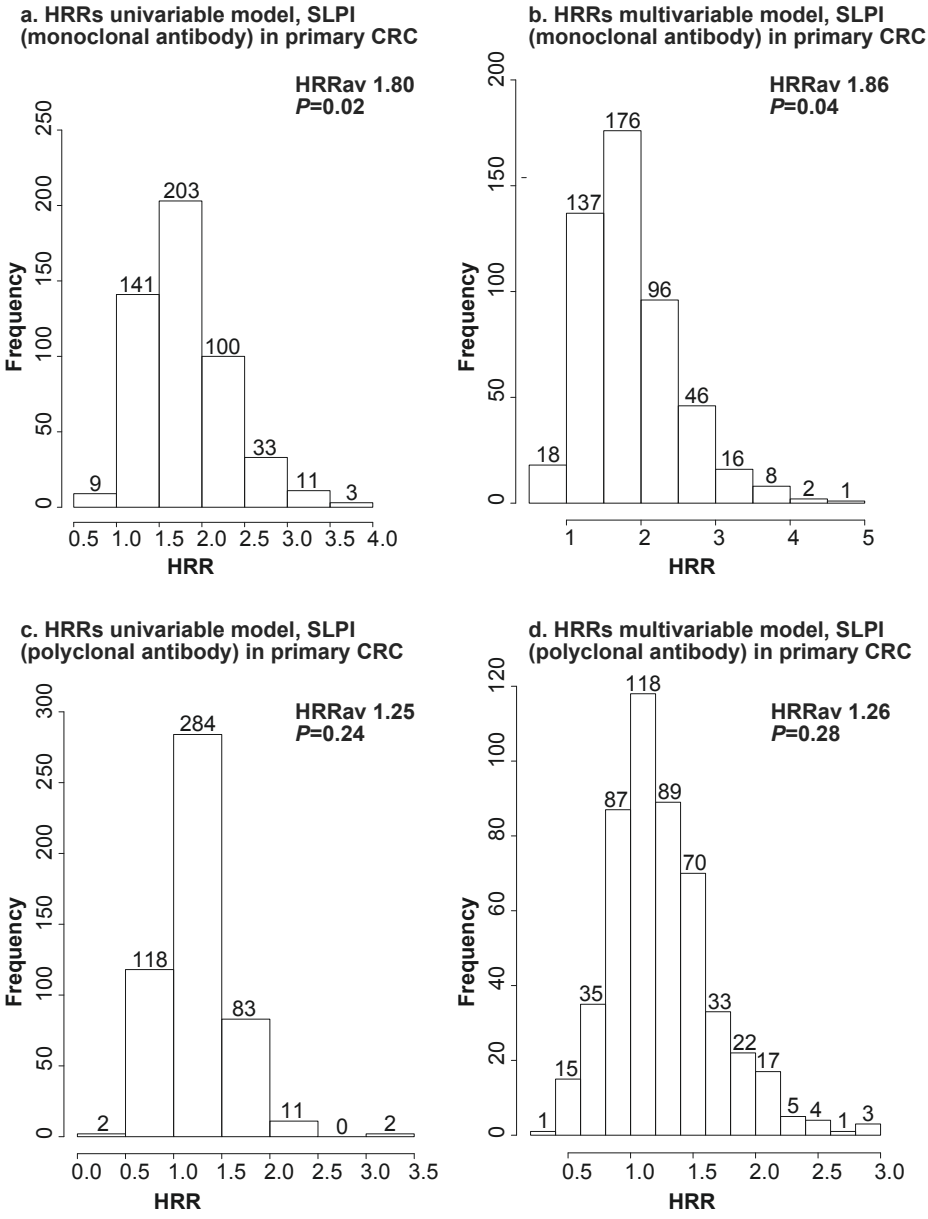
SLPI expression was scored in CRCLM tissues from 357 patients using both the monoclonal antibody and polyclonal antibody (a) and in the matched primary CRC tissues from 157 of these patients using both the monoclonal antibody and polyclonal antibody (b). Green fields represent the cases in which dichotomization into the 'SLPI-low' or 'SLPI-high' group based on the cross-validated cut-offs was not different for tissues stained using the monoclonal or polyclonal antibody (74% of patients for CRCLM and 72% of patients for primary CRC). Dotted lines represent the cut-offs for the monoclonal and polyclonal antibody. The size of the circles represents the number of patients per group, which is also indicated in the circles. Detection of SLPI using the monoclonal antibody was significantly associated with detection of SLPI using the polyclonal antibody in both CRCLM (two-sided Fisher's exact test: $P < 0.01$) and primary tumors (two-sided Fisher's exact test: $P < 0.01$).





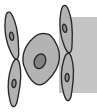
Supplementary figure 5: Relationship between SLPI expression in CRCLM and the matched primary CRC

SLPI expression in CRCLM and the matched primary tumors was compared for 140 patients based on tissues stained using the monoclonal antibody (a) and for 137 patients based on tissues stained using the polyclonal antibody (b). Green fields represent cases for which dichotomization into the 'SLPI-low' or 'SLPI-high' group based on the cross-validated cut-offs was not different between the CRCLM and matched primary CRC (55% of patients for the monoclonal antibody and 65% of patients for the polyclonal antibody). Dotted lines represent the cut-offs for the monoclonal (a) or polyclonal antibody (b). The size of the circles represents the number of patients per group, which is also indicated in the circles. SLPI expression in CRCLM was significantly associated with SLPI expression in primary tumors, as detected using the monoclonal antibody (two-sided Fisher's exact test: $P < 0.01$) or the polyclonal antibody (two-sided Fisher's exact test: $P = 0.02$).



Supplementary figure 6: Distribution of cross-validated HRRs for SLPI expression in primary CRC

The optimal cut-off for SLPI expression in primary CRC was calculated and cross-validated with 3-year overall survival after resection of liver metastases as the outcome. The distribution of HRRs is shown for SLPI scores after staining with the monoclonal antibody (a + b) and polyclonal antibody (c+d). Both univariable analyses (a + c) and multivariable analyses (b + d) are shown (see methods). HRRav = average hazard rate ratio over all 500 cycles.



CHAPTER 6

High expression of Secretory Leukocyte Protease Inhibitor (SLPI) in stage III micro-satellite stable colorectal cancer is associated with reduced disease recurrence

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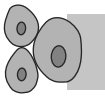
ABSTRACT

Secretory leukocyte protease inhibitor (SLPI) is a pleiotropic protein produced by healthy intestinal epithelial cells. SLPI regulates NF-κB activation, inhibits neutrophil proteases and has broad antimicrobial activity. Recently, increased SLPI expression was found in various types of carcinomas and was suggested to increase their metastatic potential. Indeed, we demonstrated that SLPI protein expression in colorectal cancer (CRC) liver metastases and matched primary tumors is associated with worse outcome, suggesting that SLPI promotes metastasis in human CRC. However, whether SLPI plays a role in CRC before distant metastases have formed is unclear. Therefore, we examined whether SLPI expression is associated with prognosis in CRC patients with localized disease. Using a cohort of 226 stage II and 160 stage III CRC patients we demonstrate that high SLPI protein expression is associated with reduced disease recurrence in patients with stage III micro-satellite stable tumors treated with adjuvant chemotherapy, independently of established clinical risk factors (hazard rate ratio 0.54, *P*-value 0.03). SLPI protein expression was not associated with disease-free survival in stage II CRC patients. Our data suggest that the role of SLPI in CRC may be different depending on the stage of disease. In stage III CRC, SLPI expression may be unfavorable for tumors, whereas SLPI expression may be beneficial for tumors once distant metastases have established.

INTRODUCTION

Secretory Leukocyte Protease Inhibitor (SLPI) is expressed and secreted mainly by human epithelial cells (1,2). SLPI maintains intestinal homeostasis by preventing tissue destruction by neutrophil proteases and by regulating the threshold of inflammatory immune responses (3-5). In the intestinal epithelium, SLPI expression is induced by repetitive microbial contact while it suppresses chemokine production by inhibiting nuclear factor kappa light chain enhancer of activated B cells (NF-κB) activation (5).

Recently, increased SLPI protein expression has been demonstrated in various types of cancer, including colorectal cancer (CRC), gastric cancer, non-small cell lung cancer and ovarian cancer (6-9). Interestingly, high tumor SLPI expression has been associated with poor prognosis. In particular, high tumor *SLPI* mRNA expression is associated with shorter overall survival in triple negative breast cancer patients (10) and gastric cancer (7). In addition, we previously showed that high SLPI protein expression in CRC liver metastases and matched primary tumors was associated with shorter overall survival (11). The precise role of SLPI in cancer is, however, unclear. Clones expressing SLPI entered the vasculature more efficiently and formed more metastases than clones that did not express SLPI in a murine model of polyclonal breast cancer (12). The role of SLPI in CRC metastasis has not extensively been studied. We previously hypothesized that SLPI expression may be beneficial for metastatic CRC tumor cells via promotion of immune evasion (11). However, SLPI has multiple diverse functions and may act differently in various processes related to tumor growth and metastasis.



In this study, we assessed whether SLPI expression in CRC at a stage prior to distant metastases formation is associated with disease recurrence. We evaluated the prognostic value of SLPI protein expression in CRC patients classified as stage II (no metastases) and stage III (metastases to regional lymph nodes, but no distant metastases). Both stage II CRC patients and stage III CRC patients have a variable prognosis and therefore the identification of prognostic factors is desired to identify which patients may benefit from adjuvant therapy (13,14). In addition, a better understanding of the biology of tumor growth and the formation of metastases may ultimately help to predict survival in CRC patients. Here, we show that high SLPI protein expression in micro-satellite stable (MSS) tumors is associated with reduced disease recurrence in stage III CRC patients treated with adjuvant chemotherapy.

METHODS

Patient cohort and tissue microarray (TMA) generation

Between 1996 and 2005, 454 CRC patients classified as stage II or stage III according to the 4th edition of the TNM-classification system underwent surgical resection of CRC at the former Kennemer Gasthuis (current Spaarne Gasthuis) hospital in Haarlem, the Netherlands. Patients with a history of colorectal cancer, patients with irradical resection of the primary tumor, patients who died within 3 months after surgery and patients who were lost for follow-up were excluded from the study cohort (Supplementary figure 1). Histologically confirmed, formaldehyde-fixed paraffin-embedded (FFPE) CRC tissue samples from 386 patients were included, as described previously (15). Tissue microarrays (TMAs) were generated from the original FFPE tissue blocks according to protocols previously described (16). In short, six tissue core biopsies of 0.6 millimeter in diameter were punched from morphologically representative tissue areas and transferred into recipient TMA paraffin blocks. Tumor samples from this cohort have previously been analyzed for micro-satellite instability (MSI) using a five-marker-based PCR analysis system, as described previously (15). For 48 out of 359 patients (13%) MSI status could not be determined. These patients were excluded from the analyses of SLPI expression in the subgroup with MSS tumors and the analyses of SLPI expression in the subgroup with MSI tumors.

SLPI immunohistochemistry

Immunohistochemistry for SLPI was performed as previously described (11). In short, sections were stained with either a monoclonal anti-human-SLPI antibody that was raised against human SLPI purified from sputum (4 µg/mL, mouse IgG1, HM2037, clone 31; HycultBiotech, Uden, The Netherlands) or a polyclonal anti-human-SLPI antibody that was raised against *Escherichia coli*-derived recombinant human SLPI (1 µg/mL, goat IgG, BAF1274; R&D Systems/Bio-Techne, Minneapolis, MN, USA). Sections were counterstained with hematoxylin (Vector Laboratories).

Previously, staining with the monoclonal antibody resulted in a clear signal with limited background staining (11). Staining with the polyclonal antibody showed more background staining, but confirmed the staining patterns observed with the monoclonal antibody (11). Therefore, in this study we focused on the results obtained with the monoclonal antibody and we used the polyclonal antibody again to support our findings.

Scoring of SLPI expression

Images of stained sections were digitally captured using an Aperio AT2 scanner (Leica Microsystems B.V., Amsterdam, The Netherlands) equipped with a 20× / 0.75 objective (UPlanSAPO; Olympus, Leiderdorp, The Netherlands). SLPI intensity in the cytoplasm of neoplastic epithelial cells was manually scored in a semi-quantitative manner as 'negative', 'weak', 'moderate' or 'strong' using the online platform Slide Score (www.slidescore.com). The scoring strategy was designed based on the range of SLPI staining intensity observed among all tissue cores and was agreed on in consultation with a pathologist. All sections were scored by the same investigator. In order to assess the reproducibility of the scoring, a second pathologist independently scored >25% of the cores with the monoclonal antibody and > 25% of the cores stained with the polyclonal antibody. Both observers were blinded to the clinical information at time of assessment. The linear weighted kappa values were 0.49 for the monoclonal antibody and 0.59 for the polyclonal antibody, indicating moderate interobserver agreement.

Statistical analysis

For both anti-SLPI antibodies, the maximum score from the one to six TMA cores stained for each patient was used in all analyses. Patients for whom none of the six cores were evaluable were excluded from the analysis (Supplementary figure 1). Disease recurrence was defined as either local tumor recurrence or distant metastases or both, as diagnosed by computed tomography or histopathology. In order to assess the prognostic value of SLPI in stage II and stage III CRC, we determined the optimal cut-offs for dichotomization of the cohort by SLPI protein expression based on 5-years disease-free survival. First, the study population was randomly divided into five subsets. The optimal cut-off for dichotomizing four-fifth of the study population was calculated using receiver operating characteristic (ROC) curve analysis for 5-year disease-free survival. This procedure was repeated five times with one-fifth of the data set varying. The cut-off most often selected was chosen as the optimal cut-off. Patients were classified as 'SLPI-low' or 'SLPI-high' based on this validated cut-off for both antibodies separately. For CRC stained with the monoclonal antibody, the optimal cut-off was negative vs weak / moderate / strong SLPI expression. For CRC stained with the polyclonal antibody, the optimal cut-off was negative / weak vs moderate / strong SLPI expression. The validated cut-offs were subsequently applied to the study population to calculate a hazard rate ratio (HRR) and 95% confidence interval for 5-year disease-free survival in univariable Cox regression analysis. In addition, a corrected HRR was calculated by multivariable Cox regression analysis for 5-year disease-free survival. The relation between SLPI expression and disease-free survival was visualized by Kaplan Meier curves. The log-rank test was used to determine whether disease-free survival varied significantly between the SLPI-low and SLPI-high

group. Categorical clinicopathological features of the SLPI-low and SLPI-high groups were compared using Pearson's Chi-squared test or Fishers exact test (two-sided) in case of expected frequencies <5. Differences between the SLPI-low and SLPI-high groups in clinicopathological features that were measured on a continuous scale and not normally distributed were compared using the Kruskal-Wallis rank sum test. The relationship between SLPI detected using the monoclonal antibody and SLPI detected using the polyclonal antibody was examined using Fisher's exact test (two-sided). All statistical analyses and visualization were performed using R version 3.5.1 (17). The 'survival' (18,19), 'survminer' (20), 'pROC' (21) and 'survivalROC' (22) packages were employed for survival analysis and to calculate the optimal cut-offs. All methods were carried out in accordance with the REMARK recommendations for reporting tumor marker prognostic studies (23).

Ethics approval and consent to participate

Collection, storage and use of the tissue samples and clinical data of the cohort were conducted in compliance with the Dutch code of conduct for responsible use of human tissue for medical research (24). Use of this cohort for the present study was evaluated and approved by the Institutional Review Board of the Netherlands Cancer Institute (IRBd18159). Collection of patient data and tissue samples was according to local and national legislation at the time of sample and data collection and in compliance with the 'Code for Proper Secondary Use of Human Tissue in The Netherlands'. This allowed the present retrospective observational study to be performed without the need for study-specific informed consent from individual patients. Experimental protocols needed for the current study were approved and the need for informed consent was waived by The Netherlands Cancer Institute, Amsterdam, The Netherlands.

RESULTS

SLPI is expressed in a subset of stage II and stage III CRC

To establish the prognostic value of SLPI in stage II and stage III CRC we assessed SLPI protein expression in CRC tissue samples from a Dutch cohort of 226 stage II and 160 stage III CRC patients. The characteristics of this study population have been described previously (15). CRC tissue samples from in total 359 patients were available for analysis of SLPI stained with the monoclonal antibody (Supplementary figure 1). We focused on the results obtained with the monoclonal antibody.

We observed SLPI expression in the cytoplasm of the tumor cells; in some tissue cores mainly on the luminal side of the tumor cells and in other tissue cores in the whole cytoplasm of the tumor cells (Figure 1a and Supplementary figure 4a). We detected

expression of SLPI in CRC in 56% of patients (Figure 1b). Using the validated cut-offs, SLPI expression was not significantly different between stage II and stage III CRC patients (Supplementary figure 2). In conclusion, we detected SLPI protein expression in a substantial subgroup of stage II and stage III CRC patients.

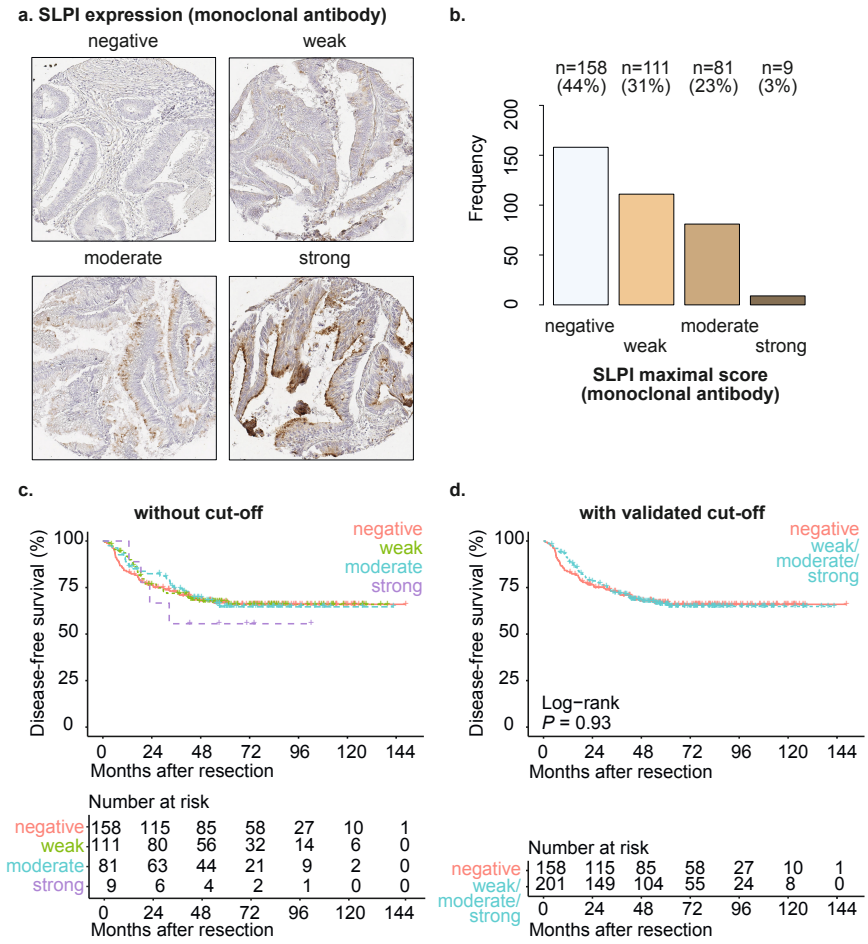


Figure 1: SLPI is expressed in a subset of stage II and stage III CRC.

Examples of TMA cores of stage II or stage III CRC stained for SLPI using the monoclonal antibody (a). Frequencies and percentages of stage II or stage III CRC scored as 'negative', 'weak', 'moderate' or 'strong' after staining with the monoclonal SLPI antibody (b); only the maximum score for each patient was included. Kaplan-Meier curves for disease-free survival after resection of the primary tumor (in months) for the total study population of stage II and stage III CRC patients stratified by SLPI expression detected using the monoclonal antibody (c and d). Curves without a cut-off (c) and with the validated cut-off (d) are shown. *P*-values were calculated using the log-rank test.

SLPI expression in the whole cohort of stage II CRC or stage III CRC is not associated with disease-free survival

When evaluating stage II and stage III CRC patients together, SLPI expression was not associated with disease-free survival (HRR 0.98, *P*-value 0.93, 95% confidence interval 0.68 – 1.42, Figures 1c and 1d). Patient age, gender, grade of differentiation of the tumor, tumor stage, nodal stage, presence of mucinous differentiation, presence of micro-satellite instability (MSI), presence of ulceration, presence of angio-invasion, occurrence of perforation, occurrence of tumor spill, treatment with adjuvant chemotherapy, disease recurrence (local or distant), CRC-related mortality, overall mortality and the follow-up time were not significantly different between patients with high or low SLPI expression in CRC tissues (Supplementary figure 2a). Patients with high SLPI expression significantly more often had left-sided tumors and significantly smaller tumors (Supplementary figure 2a).

When evaluating stage II CRC patients separately, SLPI expression was not associated with disease-free survival (HRR 1.48, *P*-value 0.19, 95% confidence interval 0.81 – 2.69, Figure 2a). When evaluating stage III CRC patients separately, SLPI expression was also not associated with disease-free survival (HRR 0.70, *P*-value 0.13, 95% confidence interval 0.43 – 1.12, Figure 2b). There were no significant differences in clinicopathological characteristics in stage II or stage III patients between the low-SLPI and the high-SLPI group (Supplementary figure 2b and 2c).

High expression of SLPI in stage III micro-satellite stable CRC is associated with reduced disease recurrence

Because patients with micro-satellite instable (MSI) CRC are known to have a better prognosis compared to patients with micro-satellite stable (MSS) CRC (25-27), we evaluated the prognostic value of SLPI in the subgroup of patients with MSS tumors and in the subgroup of patients with MSI tumors separately. In stage II patients with MSS tumors, SLPI expression was not associated with disease-free survival (HRR 1.40, *P*-value 0.31, 95% confidence interval 0.73 – 2.68, Figure 2c). In stage II patients with MSI tumors, SLPI expression was also not associated with disease-free survival (HRR 1.77, *P*-value 0.62, 95% confidence interval 0.18 – 17.01, Figure 2e).

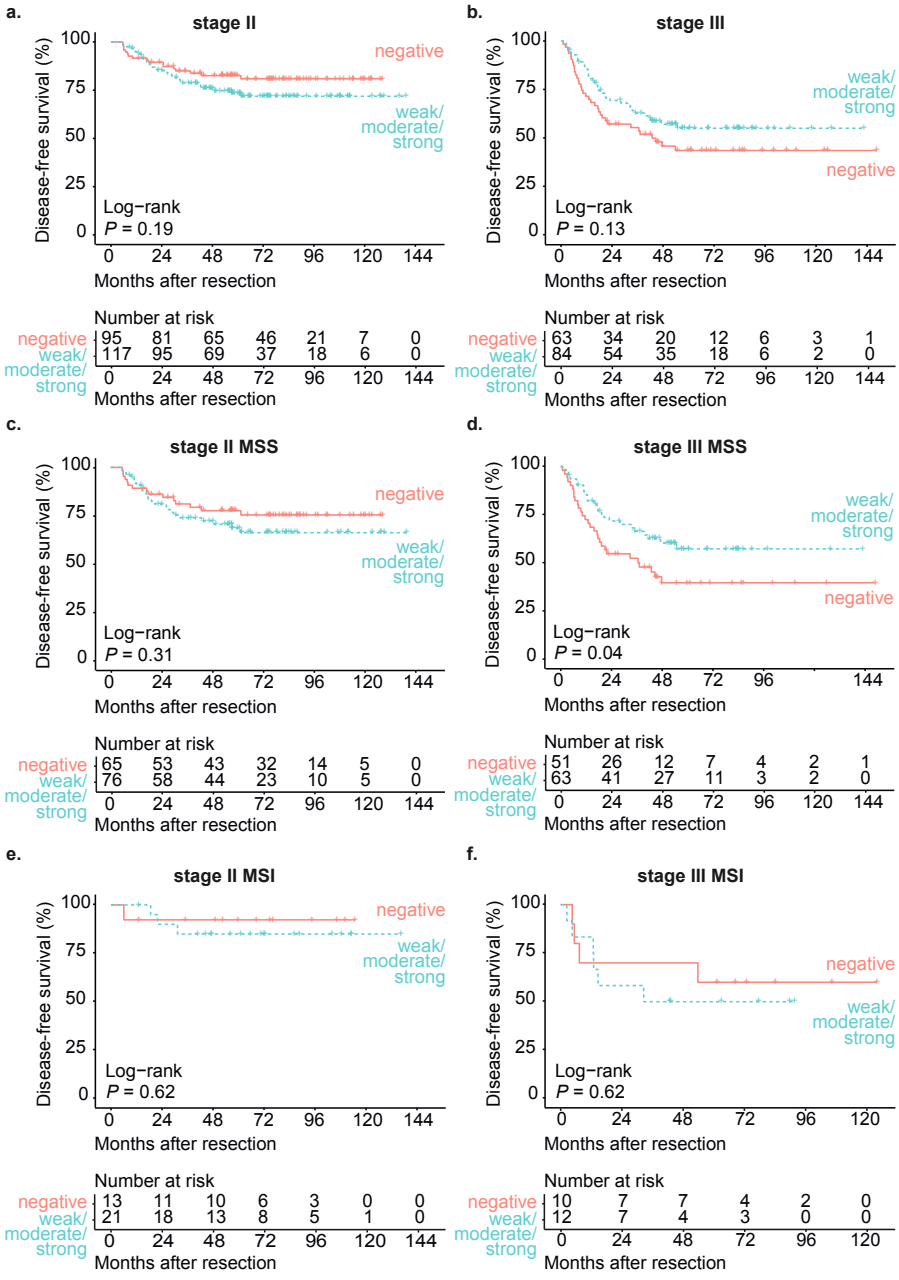


Figure 2: High SLPI expression in stage III MSS CRC is associated with increased disease-free survival.

Kaplan-Meier curves for disease-free survival after resection of the primary tumor (in months) for either stage II CRC patients (a) or stage III CRC patients (b) stratified by SLPI expression detected using the monoclonal antibody. Kaplan-Meier curves for disease-free survival after resection of the primary tumor (in months) for stage II CRC patients with MSS (c) or MSI tumors (e) and stage III CRC patients with MSS (d) or MSI tumors (f) stratified by SLPI expression detected using the monoclonal antibody. Curves with the validated cut-off are shown. *P*-values were calculated using the log-rank test. MSS = micro-satellite stable. MSI = micro-satellite instable.

In contrast, in stage III patients with MSS tumors high SLPI expression was associated with a significantly increased disease-free survival (HRR 0.58, *P*-value 0.04, 95% confidence interval 0.34 – 0.99, Figure 2d). Mucinous differentiation was significantly more often present in stage III patients with MSS tumors in the SLPI-high group compared to the SLPI-low group (*P*-value <0.01, Supplementary figure 2d). Mucinous differentiation was not associated with disease-free survival in this subgroup (HRR 0.99, *P*-value 0.98, 95% confidence interval 0.49 – 2.02) and is therefore not likely to confound the association between SLPI and disease-free survival in stage III patients with MSS tumors. In stage III CRC patients with MSI tumors, SLPI expression was not associated with disease-free survival (HRR 1.38, *P*-value 0.62, 95% confidence interval 0.39 – 4.93, Figure 2f). In conclusion, high SLPI expression is associated with reduced disease recurrence in stage III CRC patients with MSS tumors.

High expression of SLPI in micro-satellite stable CRC is associated with reduced disease recurrence in stage III patients treated with adjuvant chemotherapy

As a substantial group of stage III CRC patients in this cohort has been treated with 5FU-based adjuvant chemotherapy, which is likely to affect disease-free survival, we evaluated the association between SLPI and disease-free survival in patients with MSS tumors separately for patients who did and who did not receive adjuvant chemotherapy. When we stratified for adjuvant chemotherapy in the whole cohort of stage II and stage III MSS CRC, SLPI expression was not associated with disease-free survival in patients who did not receive adjuvant chemotherapy (HRR 0.93, *P*-value 0.80, 95% confidence interval 0.54 – 1.60, Figure 3a) or in patients who received adjuvant chemotherapy (HRR 0.81, *P*-value 0.51, 95% confidence interval 0.43 – 1.52, Figure 3b).

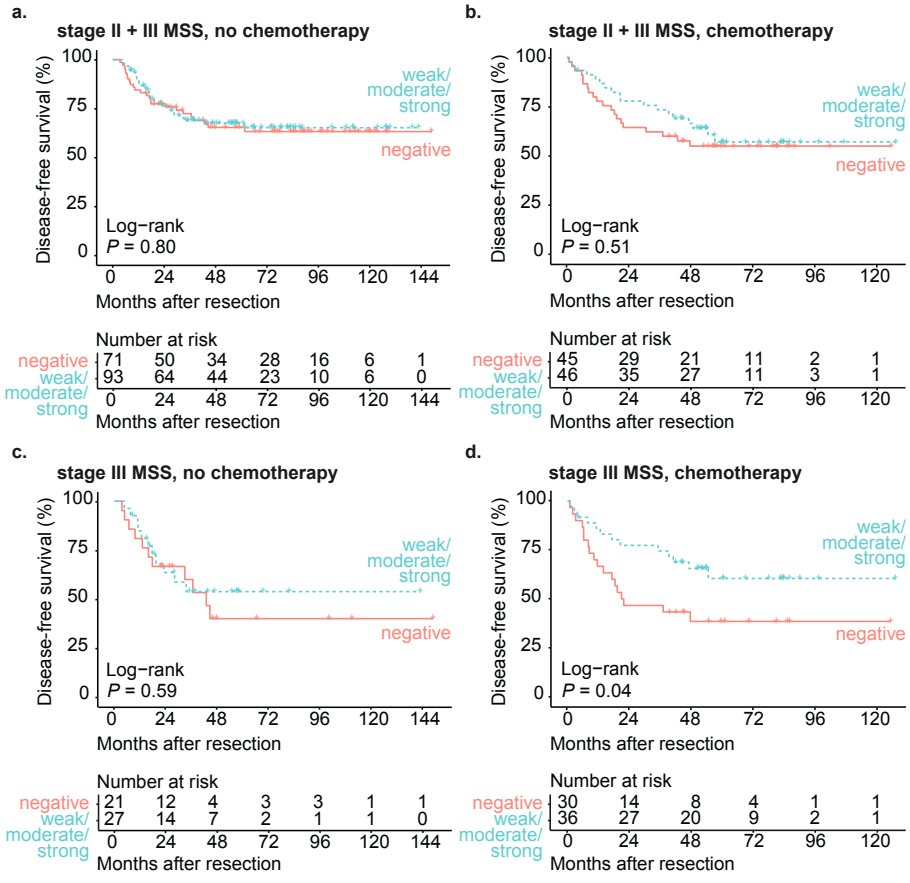


Figure 3: High SLPI expression in chemotherapy-treated stage III MSS CRC patients is associated with increased disease-free survival.

Kaplan-Meier curves for disease-free survival after resection of the primary tumor (in months) for stage II and stage III CRC patients with MSS tumors not treated with adjuvant chemotherapy (a) or treated with adjuvant chemotherapy (b) stratified by SLPI expression detected using the monoclonal antibody. Kaplan-Meier curves for disease-free survival after resection of the primary tumor (in months) for stage III CRC patients with MSS tumors not treated with adjuvant chemotherapy (c) or treated with adjuvant chemotherapy (d) stratified by SLPI expression detected using the monoclonal antibody. Curves with the validated cut-off are shown. *P*-values were calculated using the log-rank test. MSS = micro-satellite stable

In stage III patients with MSS tumors who did not receive adjuvant chemotherapy, SLPI expression was not significantly associated with disease-free survival (HRR 0.80, *P*-value 0.59, 95% confidence interval 0.34 – 1.84, Figure 3c). To determine whether SLPI has prognostic value in stage III MSS CRC patients who received adjuvant chemotherapy, we assessed disease free survival in patients with low SLPI expression and high SLPI expression in the 58% of stage III MSS CRC patients who received

adjuvant chemotherapy (Figure 3c and 3d). In stage III MSS patients who received adjuvant chemotherapy, high SLPI expression was significantly associated with increased disease-free survival (HRR 0.48, *P*-value 0.04, 95% confidence interval 0.23 – 0.98, Figure 3d). In these patients, clinicopathological characteristics were not different between the low-SLPI and the high-SLPI group (Supplementary figure 2e). These findings raised the question whether treatment with adjuvant chemotherapy could explain the association between high SLPI expression and reduced disease recurrence in stage III MSS CRC patient. However, treatment with adjuvant chemotherapy was not significantly associated with disease recurrence in stage III MSS colorectal cancer patients (HRR 1.04, P-value 0.89, 95% confidence interval 0.61 - 1.78) and the proportion of patients who received adjuvant chemotherapy was not different between the low-SLPI and the high-SLPI group (Supplementary figure 2d). Therefore, the association between high SLPI expression in stage III MSS colorectal cancer and reduced disease recurrence cannot be explained by treatment with adjuvant chemotherapy.

In conclusion, high SLPI expression is associated with reduced disease recurrence in stage III patients with MSS tumors who did receive adjuvant chemotherapy, but not in stage III patients with MSS tumors who did not receive adjuvant chemotherapy.

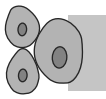
SLPI expression has prognostic value in stage III patients with MSS CRC independently of established clinical risk factors

Next, we investigated whether the prognostic value of SLPI expression in stage III CRC patients with MSS tumors was independent of previously established clinical risk factors. The following factors have been previously demonstrated to be associated with prognosis in CRC patients: tumor location, tumor stage, nodal stage, isolated tumor deposits, angio-invasion, tumor histological grade, ulceration, perforation and tumor spill (28-30).

In stage III patients with MSS tumors, the following factors were associated with disease recurrence in the univariable Cox regression with a *P*-value of 0.1 or below and were therefore included in the multivariable Cox regression model: tumor stage (HRR 1.86, *P*-value 0.01, 95% confidence interval 1.16 – 3.00), nodal stage (HRR 1.57, *P*-value 0.1, 95% confidence interval 0.91 – 2.71), isolated tumor deposits (HRR 1.86, *P*-value 0.03, 95% confidence interval 1.05 – 3.29), angio-invasion (HRR 2.65, *P*-value <0.01, 95% confidence interval 1.56 – 4.49), tumor histological grade (HRR 1.80, *P*-value 0.05, 95% confidence interval 1.00 – 3.25) and perforation (HRR 2.21, *P*-value 0.03, 95% confidence interval 1.04 – 4.68). The prognostic value of SLPI expression was not confounded by these factors as high SLPI expression was associated with reduced disease recurrence in the multivariable model (HRR 0.54, *P*-value 0.03, 95% confidence

interval 0.31 – 0.94). Factors that were significantly associated with disease recurrence in this model were angio-invasion (HRR 2.88, *P*-value <0.01, 95% confidence interval 1.53 – 5.42) and perforation (HRR 3.04, *P*-value <0.01, 95% confidence interval 1.38 – 6.70). In conclusion, high SLPI expression in MSS tumors of stage III CRC patients was associated with significantly reduced disease recurrence after surgical resection of the primary tumor, independently of known clinical risk factors.

In addition, we evaluated whether SLPI expression also had prognostic value independently of clinical risk factors in stage III CRC patients with MSS tumors who received adjuvant chemotherapy, as in these patients high SLPI expression was associated with reduced disease recurrence. In this subgroup, the following clinical risk factors were associated with disease recurrence in the univariable Cox regression with a *P*-value of 0.1 or below and were therefore included in the multivariable Cox regression model: tumor stage (HRR 1.96, *P*-value 0.03, 95% confidence interval 1.08 – 3.57), nodal stage (HRR 1.77, *P*-value 0.1, 95% confidence interval 0.90 – 3.48), isolated tumor deposits (HRR 1.96, *P*-value 0.06, 95% confidence interval 0.97 – 3.97), angio-invasion (HRR 3.51, *P*-value < 0.01, 95% confidence interval 1.76 – 7.00), tumor histological grade (HRR 1.74, *P*-value 0.1, 95% confidence interval 0.82 – 3.70), ulceration (HRR 0.40, *P*-value 0.02, 95% confidence interval 0.18 – 0.86), perforation (HRR 2.84, *P*-value 0.07, 95% confidence interval 0.87 – 9.34) and tumor spill (HRR 14.18, *P*-value <0.01, 95% confidence interval 2.73 – 73.6). The prognostic value of SLPI expression was not confounded by these factors (HRR 0.43, *P*-value 0.03, 95% confidence interval 0.20 – 0.93). The factors that remained significantly associated with disease recurrence in this model were angio-invasion (HRR 3.13, *P*-value 0.01, 95% confidence interval 1.29 – 7.61) and perforation (HRR 4.49, *P*-value 0.04, 95% confidence interval 1.11 – 18.13). In conclusion, detection of high SLPI expression in MSS tumors of stage III CRC patients who received adjuvant chemotherapy was associated with significantly reduced disease recurrence after surgical resection the primary tumor, independently of known clinical risk factors.



Detection of SLPI with the polyclonal antibody supports the association between high expression of SLPI in stage III micro-satellite stable CRC and reduced disease recurrence

To robustly assess the prognostic value of SLPI, we detected SLPI protein expression by immunohistochemistry using both a monoclonal antibody and a polyclonal antibody. Previously, we observed a significant association between SLPI detected with the monoclonal antibody and SLPI detected with the polyclonal antibody (11). The monoclonal antibody had better discriminative potential and revealed a stronger association between SLPI and prognosis in patients with CRC liver metastases, and

results obtained with the polyclonal antibody confirmed our findings (11). Therefore, we also assessed in the current study whether SLPI staining with the polyclonal antibody revealed the association between high expression of SLPI in MSS tumors and reduced disease recurrence in stage III CRC patients.

Using the polyclonal antibody, we detected expression of SLPI in the cytoplasm of tumor cells of 96% of CRC patients (Supplementary figure 4a and 4b). We compared the scores for SLPI expression stained with the monoclonal antibody with the scores for SLPI expression stained with the polyclonal antibody in 349 patients (Supplementary figure 3). There was a significant association between detection of high SLPI expression with the monoclonal antibody and detection of high SLPI expression with the polyclonal antibody (two-sided Fisher's exact test: *P*-value < 0.01). Both staining with the monoclonal and polyclonal antibody resulted in the same classification as either SLPI-low or SLPI-high for 77% of patients, based on the validated cut-offs (Supplementary figure 3). SLPI expression scores were more frequently lower in CRC stained with the monoclonal antibody compared to the polyclonal antibody than vice versa, which indicates that the monoclonal antibody has a higher threshold of detection for SLPI, as we observed previously (11).

Detection of SLPI using the polyclonal antibody led to the same trends as the data obtained with the monoclonal antibody. SLPI expression detected with the polyclonal antibody was not associated with disease-free survival in the whole cohort of stage II and stage III CRC patients (HRR 0.91, *P*-value 0.60, 95% confidence interval 0.63 – 1.31, Supplementary figure 4c and 4d) or in stage II patients separately (HRR 1.16, *P*-value 0.61, 95% confidence interval 0.65 – 2.08, Supplementary figure 5a). In stage III CRC patients, we observed a non-significant trend between high SLPI expression and increased disease-free survival (HRR 0.70, *P*-value 0.14, 95% confidence interval 0.44 – 1.13, Supplementary figure 5b). In stage III patients with MSS tumors, we also observed a trend between high SLPI expression detected with the polyclonal antibody and increased disease-free survival (HRR 0.62, *P*-value 0.08, 95% confidence interval 0.36 – 1.06, Supplementary figure 5d). Distant disease recurrence was significantly more frequent in the SLPI-low group when SLPI was detected with the polyclonal antibody (Supplementary figure 7d), which fits with the association between high SLPI expression and increased disease-free survival. We observed similar results in stage III patients with MSS tumors who received adjuvant chemotherapy (Supplementary figure 6d and Supplementary figure 7e).

DISCUSSION

SLPI is expressed by healthy intestinal epithelial cells throughout the body and maintains tissue homeostasis by preventing protease-induced tissue damage and by regulating inflammatory immune responses. We previously demonstrated that high SLPI expression in CRC liver metastases and matched primary tumors of patients with colorectal liver metastases is associated with poorer overall survival (11). However, the prognostic value of SLPI in CRC patients with localized disease has not been established. Here, we studied SLPI protein expression in a large cohort of stage II and stage III CRC patients and found that high SLPI protein expression in MSS tumors is associated with reduced disease recurrence after resection of the primary tumor in stage III CRC patients. In particular, high SLPI expression was associated with reduced disease recurrence in stage III MSS CRC patients treated with 5FU-based adjuvant chemotherapy, independently of established clinical risk factors.

Previously, we observed that high SLPI expression in liver metastases and matched primary tumors is associated with worse prognosis (11). The data presented in the current study may appear contradictive, as we find an association between high SLPI expression and reduced disease recurrence in stage III CRC patients, while recurrence most often involves distant metastasis formation. In addition, we do not find an association between SLPI expression and disease recurrence in stage II CRC patients. Our data therefore argue that regulation of SLPI expression or its function in CRC may vary depending on the stage of the disease. In the current study, primary tumor tissue was obtained from patients with lymph node metastases prior to formation of distant metastases. At this stage, SLPI expression in the primary tumor appears to reflect a biological process which is unfavorable to the tumor and is associated with reduced formation of distant metastases at a later time. In contrast, in the previous cohort all patients developed liver metastases and in a substantial number of patients liver metastases were found at the same time or within 6 months after detection of the primary tumor (31). The primary tumors of patients in the liver metastases cohort are therefore likely at a more advanced stage compared to the primary tumors of stage III patients in the current study. In addition, in the previous study we assessed overall survival after resection of liver metastases, whereas in the current study the outcome was defined as either local disease recurrence, development of distant metastases or both. It would be interesting to investigate whether SLPI expression in the current cohort is associated with poorer survival after the disease has recurred, but the subgroup of patients with disease recurrence was too low to have sufficient power to assess this. Thus, we studied different steps in the progression of CRC in

this cohort and our previous cohort and we show that SLPI expression may relate to different biological processes or have a different consequence for the tumor.

There are multiple possible explanations for our findings. Firstly, tumor cells expressing SLPI in stage III patients may be different from the tumor cells which later form metastases and were studied in our CRC liver metastases cohort. In differentiated tumor cells SLPI expression may reflect a response to the tumor microenvironment, whereas in more aberrant cells SLPI expression may reflect increased metastatic potential. Unfortunately, based on our current analyses we cannot assess whether SLPI-expressing tumor cells from stage III patients are indeed different from those in the primary tumors of patients with liver metastases. Of note, we did observe that SLPI expression in MSS tumors from stage III patients is associated with mucinous differentiation, but in the CRC liver metastases cohort mucinous differentiation was not assessed.

Alternatively, irrespective of whether there is a difference in the tumor cells expressing SLPI, SLPI may exert opposing functions during the course of disease. Although stage III CRC patients have lymph node metastases, tumor cells need to acquire different functionalities for the formation of distant metastases and therefore the role of SLPI in this stage could be different. For example, during distant metastasis formation SLPI can induce the formation of vessel-like structures which provide a blood supply to hypoxic regions of the tumor (12). Moreover, SLPI acts as an anticoagulant (12) and inhibits the antiangiogenic factor Endostatin (32). SLPI may also promote vascular invasion via induction of MMP-2 and MMP-9 production by tumor cells (10). These tumor cell functions may not yet be active in primary tumor cells in stage III CRC patients. In contrast, in the stage prior to distant metastasis formation SLPI expression in the tumor may counteract other tumor cell functions. For example, SLPI can inhibit tumor growth, suppress migration and promote apoptosis *in vitro* (33-36). However, the precise role of SLPI in the different steps of CRC metastasis formation remains to be investigated.

High SLPI expression was only associated with reduced disease recurrence in stage III CRC patients with MSS tumors, in contrast to patients with MSI tumors. The reason for this specific association is unclear. MSI tumors are known to elicit strong anti-tumor immune responses relative to MSS tumors (37). SLPI expression can be modulated by inflammation and ensuing toll-like receptor signaling. As such, it could have been expected that SLPI expression is higher in MSI tumors and also impacts disease recurrence in this group of patients. However, MSI status was not associated with the degree of SLPI expression in tumors of stage II or stage III patients. Possibly,

inhibition of NF-κB by SLPI is more relevant in MSS tumors compared to MSI tumors, as dedifferentiation of tumor cells due to NF-κB-induced Wnt-signaling may particularly occur in MSS CRC (38).

We only observed the association between high SLPI expression in MSS tumors and reduced disease recurrence in stage III patients who did receive adjuvant chemotherapy (Figure 3d) and not in patients who did not receive adjuvant chemotherapy (Figure 3c). This suggests that high SLPI expression may predispose to better killing of tumor cells by 5FU-based adjuvant chemotherapy. Possibly, the formation of vessel-like structures and anticoagulation induced by SLPI (12) make hypoxic regions of the tumor more accessible to chemotherapy.

In conclusion, high SLPI expression in MSS CRC is associated with reduced disease recurrence after resection of the primary tumor and adjuvant chemotherapy in stage III patients. In view of the association between high SLPI expression in CRC liver metastases and matched primary tumors and poorer overall survival, these data suggest different roles for SLPI in CRC before and after the formation of distant metastases. In addition, high SLPI expression in MSS tumors may predict a better response to adjuvant chemotherapy in stage III CRC patients.

FUNDING

SN was funded by the Dutch Digestive Foundation (grant registration number: Focus Project 15-17; https://www.mlds.nl). JNS received the grant. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

ACKNOWLEDGEMENTS

We thank Eric J. T. Belt for the collection of clinicopathological data and tissue samples from the patients in this cohort, Jan Hudeček for developing and making Slide Score available to us, Menno de Vries for managing the clinical database, and Dimitris Rizopoulos for advice on the statistical analyses.

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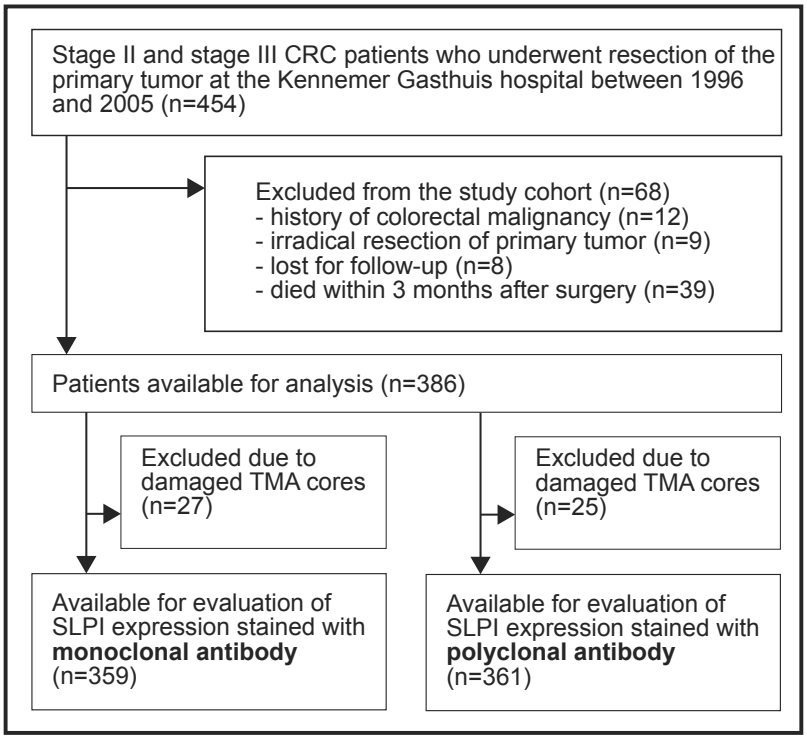
Study flow diagram



Supplementary figure 1: Flow diagram of the study cohort.

In total, 454 patients with stage II or stage III CRC underwent resection of the primary tumor at the Kennemer Gasthuis hospital between 1996 and 2005. Patients with a history of colorectal cancer, patients with an irradical resection of the primary tumor, patients who died within 3 months after the resection and patients who were lost for follow-up were excluded, resulting in a study cohort of 386 patients. After excluding patients for whom none of the six cores could be scored due to technical reasons, SLPI expression was scored in CRC stained with the monoclonal antibody from 359 patients and CRC stained with the polyclonal antibody from 361 patients.

a. Characteristics of stage II and stage III patients included in the analysis of SLPI expression using the monoclonal antibody

Clinicopathological variable	**SLPI-low (n=158)**	**SLPI-high (n=201)**	**Total (n=359)**	***P*-value**
Age in years, median (min - max)	71 (28 – 92)	73 (35 – 92)	72 (28 – 92)	0.26[1]
Gender female male	70 (44%) 88 (56%)	99 (49%) 102 (52%)	169 (47%) 190 (53%)	0.35[2]
Tumor location right (cecum until flexura lienalis) left (flexura lienalis to rectum)	79 (50%) 79 (50%)	79 (39%) 122 (61%)	158 (44%) 201 (56%)	**0.04[2]**
Tumor histological grade well-differentiated moderately differentiated poorly differentiated	8 (5%) 199 (75%) 31 (20%)	12 (6%) 164 (82%) 25 (12%)	20 (6%) 283 (79%) 56 (16%)	0.17[2]
Tumor maximal diameter in mm, median (min - max)	40 (15 – 130)	35 (10 – 100)	40 (10 – 130)	**0.03[1]**
Stage II III	95 (60%) 63 (40%)	117 (58%) 84 (42%)	212 (59%) 147 (41%)	0.71[2]
Tumor stage T1 T2 T3 T4	2 (1%) 5 (3%) 134 (84%) 17 (11%)	2 (1%) 14 (7%) 169 (84%) 16 (8%)	4 (1%) 19 (5%) 303 (84%) 33 (9%)	0.34[3]
Nodal stage (Stage III patients only) N1 N2	40 (64%) 23 (37%)	60 (71%) 24 (29%)	100 (68%) 47 (32%)	0.31[2]
Mucinous differentiation	27 (17%)	48 (24%)	75 (21%)	0.12[2]
MSI-status MSI MSS Unknown	23 (15%) 116 (73%) 19 (12%)	33 (16%) 139 (69%) 29 (14%)	56 (16%) 255 (71%) 48 (13%)	0.55[2]
Ulceration	125 (79%)	152 (76%)	277 (77%)	0.43[2]
Angio-invasion	34 (22%)	36 (18%)	70 (20%)	0.39[2]
Emergency surgery	21 (13%)	26 (13%)	47 (13%)	0.92[2]
Perforation (pre-/per-/post-operative)	12 (8%)	19 (10%)	31 (9%)	0.53[2]
Tumor spill	4 (3%)	8 (4%)	12 (3%)	0.45[2]
Adjuvant chemotherapy	52 (33%)	64 (32%)	116 (32%)	0.83[2]
Disease recurrence	51 (32%)	65 (32%)	116 (32%)	0.99[2]
Local disease recurrence	14 (9%)	25 (12%)	39 (11%)	0.28[2]
Distant disease recurrence	45 (29%)	51 (25%)	96 (27%)	0.51[2]

a. (Continued)

Clinicopathological variable	SLPI-low (n=158)	SLPI-high (n=201)	Total (n=359)	*P*-value
CRC-related mortality	42 (27%)	49 (24%)	91 (25%)	0.63[2]
Overall mortality	65 (41%)	100 (50%)	165 (46%)	0.10[2]
Follow-up time in months, median (min – max)	59.7 (3.4 – 148.6)	57.1 (4.1 – 142.6)	57.3 (3.4 – 148.6)	0.50[1]

[1]Kruskal-Wallis rank sum test
[2]Pearson's Chi-squared test
[3]Fisher's exact test

b. Characteristics of stage II patients included in the analysis of SLPI expression using the monoclonal antibody

Clinicopathological variable	SLPI-low (n=95)	SLPI-high (n=117)	Total (n=212)	*P*-value
Age in years, median (min - max)	71 (28 – 92)	74 (40 – 92)	73 (28 – 92)	0.13[1]
Gender female male	42 (44%) 53 (56%)	62 (53%) 55 (47%)	104 (49%) 108 (51%)	0.20[2]
Tumor location right (cecum until flexura lienalis) left (flexura lienalis to rectum)	45 (47%) 50 (53%)	46 (39%) 71 (61%)	91 (43%) 121 (57%)	0.24[2]
Tumor histological grade well-differentiated moderately differentiated poorly differentiated	7 (7%) 72 (76%) 16 (17%)	9 (8%) 97 (83%) 11 (9%)	16 (8%) 169 (80%) 27 (13%)	0.27[2]
Tumor maximal diameter in mm, median (min - max)	40 (20 – 130)	40 (10 -100)	40 (10 – 130)	0.18[1]
Tumor stage T3 T4	83 (87%) 12 (13%)	107 (92%) 10 (9%)	190 (90%) 22 (10%)	0.33[2]
Mucinous differentiation	17 (18%)	27 (23%)	44 (21%)	0.36[2]
MSI-status MSI MSS Unknown	13 (14%) 65 (68%) 17 (18%)	21 (18%) 76 (65%) 20 (17%)	34 (16%) 141 (67%) 37 (18%)	0.41[2]
Ulceration	73 (77%)	87 (74%)	160 (76%)	0.68[2]
Angio-invasion	14 (15%)	8 (7%)	22 (10%)	0.06[2]
Emergency surgery	11 (12%)	14 (12%)	25 (12%)	0.93[2]
Perforation (pre-/per-/post-operative)	7 (7%)	13 (11%)	20 (9%)	0.35[2]
Tumor spill	3 (3%)	3 (3%)	6 (3%)	1.00[3]
Adjuvant chemotherapy	17 (18%)	16 (13%)	33 (16%)	0.40[2]
Disease recurrence	17 (18%)	30 (26%)	47 (22%)	0.18[2]
Local disease recurrence	6 (6%)	15 (13%)	21 (10%)	0.12[2]
Distant disease recurrence	16 (17%)	21 (18%)	37 (18%)	0.83[2]
CRC-related mortality	15 (16%)	20 (17%)	35 (17%)	0.80[2]
Overall mortality	31 (33%)	53 (45%)	84 (40%)	0.06[2]
Follow-up time in months, median (min – max)	73.8 (9.0 – 128.4)	61.0 (5.3 – 139.6)	63.5 (5.3 – 139.6)	0.20[1]

[1]Kruskal-Wallis rank sum test
[2]Pearson's Chi-squared test
[3]Fisher's exact test

c. Characteristics of stage III patients included in the analysis of SLPI expression using the monoclonal antibody

Clinicopathological variable	SLPI-low (n=63)	SLPI-high (n=84)	Total (n=147)	*P*-value
Age in years, median (min - max)	71 (34 – 87)	73 (35 – 91)	72 (34 – 91)	0.99[1]
Gender female male	28 (44%) 35 (56%)	37 (44%) 47 (56%)	65 (44%) 82 (56%)	0.96[2]
Tumor location right (cecum until flexura lienalis) left (flexura lienalis to rectum)	34 (54%) 29 (46%)	33 (39%) 51 (61%)	67 (46%) 80 (54%)	0.08[2]
Tumor histological grade well-differentiated moderately differentiated poorly differentiated	1 (2%) 47 (75%) 15 (24%)	3 (4%) 67 (80%) 14 (17%)	4 (3%) 114 (78%) 29 (20%)	0.52[3]
Tumor maximal diameter in mm, median (min - max)	40 (15 – 80)	35 (10 – 100)	40 (10 – 100)	0.09[1]
Tumor stage T1 T2 T3 T4	2 (3%) 5 (8%) 51 (81%) 5 (8%)	2 (2%) 14 (17%) 62 (74%) 6 (7%)	4 (3%) 19 (13%) 113 (77%) 11 (8%)	0.48[3]
Nodal stage N1 N2	40 (64%) 23 (37%)	60 (71%) 24 (29%)	100 (68%) 47 (32%)	0.31[2]
Mucinous differentiation	10 (16%)	21 (25%)	31 (21%)	0.18[2]
MSI-status MSI MSS Unknown	10 (16%) 51 (81%) 2 (3%)	12 (14%) 63 (75%) 9 (11%)	22 (15%) 114 (78%) 11 (8%)	0.95[2]
Ulceration	52 (83%)	65 (77%)	177 (80%)	0.44[2]
Angio-invasion	20 (32%)	28 (33%)	48 (33%)	0.84[2]
Emergency surgery	10 (16%)	12 (14%)	22 (15%)	0.79[2]
Perforation (pre-/per-/post-operative)	5 (8%)	6 (7%)	11 (8%)	1.00[3]
Tumor spill	1 (2%)	5 (6%)	6 (4%)	0.24[3]
Adjuvant chemotherapy	35 (56%)	48 (57%)	83 (57%)	0.85[2]
Disease recurrence	34 (54%)	35 (42%)	69 (47%)	0.14[2]
Local disease recurrence	8 (13%)	10 (12%)	18 (12%)	0.89[2]
Distant disease recurrence	29 (46%)	30 (36%)	59 (40%)	0.21[2]
CRC-related mortality	27 (43%)	29 (35%)	56 (38%)	0.30[2]
Overall mortality	34 (54%)	47 (56%)	81 (55%)	0.81[2]
Follow-up time in months, median (min – max)	45.7 (3.4 – 148.6)	52.2 (4.1 – 142.6)	50.4 (3.4 – 148.6)	0.55[1]

[1]Kruskal-Wallis rank sum test
[2]Pearson's Chi-squared test
[3]Fisher's exact test

d. Characteristics of stage III patients with MSS tumors included in the analysis of SLPI expression using the monoclonal antibody

Clinicopathological variable	SLPI-low (n=51)	SLPI-high (n=63)	Total (n=114)	*P*-value
Age in years, median (min - max)	71 (37 – 87)	72 (38 – 91)	72 (37 – 91)	0.89[1]
Gender female male	18 (35%) 33 (65%)	28 (44%) 35 (56%)	46 (40%) 68 (60%)	0.32[2]
Tumor location right (cecum until flexura lienalis) left (flexura lienalis to rectum)	24 (47%) 27 (53%)	24 (38%) 39 (62%)	48 (42%) 66 (58%)	0.34[2]
Tumor histological grade well-differentiated moderately differentiated poorly differentiated	1 (2%) 42 (82%) 8 (16%)	3 (5%) 51 (81%) 9 (14%)	4 (4%) 93 (82%) 17 (15%)	0.85[3]
Tumor maximal diameter in mm, median (min - max)	40 (15 – 80)	30 (10 – 100)	35 (10 – 100)	0.19[1]
Tumor stage T1 T2 T3 T4	2 (4%) 5 (10%) 41 (80%) 3 (6%)	2 (3%) 12 (19%) 44 (70%) 5 (8%)	4 (4%) 17 (15%) 85 (75%) 8 (7%)	0.49[3]
Nodal stage N1 N2	33 (65%) 18 (35%)	46 (73%) 17 (27%)	79 (69%) 35 (31%)	0.34[2]
Mucinous differentiation	3 (6%)	16 (25%)	19 (17%)	**<0.01[2]**
Ulceration	42 (82%)	48 (76%)	90 (79%)	0.42[2]
Angio-invasion	17 (33%)	20 (32%)	37 (33%)	0.86[2]
Emergency surgery	9 (18%)	9 (14%)	18 (16%)	0.63[2]
Perforation (pre-/per-/post-operative)	5 (10%)	5 (8%)	10 (9%)	0.75[3]
Tumor spill	1 (2%)	4 (6%)	5 (4%)	0.38[3]
Adjuvant chemotherapy	30 (59%)	36 (57%)	66 (58%)	0.86[2]
Disease recurrence	29 (57%)	24 (38%)	53 (47%)	0.05[2]
Local disease recurrence	6 (12%)	8 (13%)	14 (12%)	0.88[2]
Distant disease recurrence	25 (49%)	20 (32%)	45 (40%)	0.06[2]
CRC-related mortality	23 (45%)	22 (35%)	45 (40%)	0.27[2]
Overall mortality	30 (59%)	37 (59%)	67 (59%)	0.99[2]
Follow-up time in months, median (min – max)	44.9 (3.4 – 148.6)	52.5 (4.1 – 142.6)	46.9 (3.4 – 148.6)	0.51[1]

[1]Kruskal-Wallis rank sum test
[2]Pearson's Chi-squared test
[3]Fisher's exact test

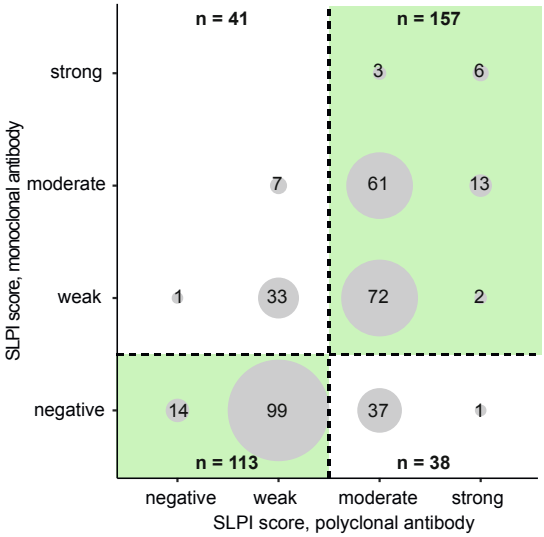
e. Characteristics of stage III patients with MSS tumors who received adjuvant chemotherapy and were included in the analysis of SLPI expression using the monoclonal antibody

Clinicopathological variable	SLPI-low (n=30)	SLPI-high (n=36)	Total (n=66)	*P*-value
Age in years, median (min - max)	69 (37 – 83)	66 (38 – 82)	67 (37 – 83)	0.79[1]
Gender female male	8 (27%) 22 (73%)	13 (36%) 23 (64%)	21 (32%) 45 (68%)	0.41[2]
Tumor location right (cecum until flexura lienalis) left (flexura lienalis to rectum)	15 (50%) 15 (50%)	14 (39%) 22 (61%)	29 (44%) 37 (56%)	0.37[2]
Tumor histological grade well-differentiated moderately differentiated poorly differentiated	0 (0%) 25 (83%) 5 (17%)	2 (6%) 28 (78%) 6 (17%)	2 (3%) 53 (80%) 11 (17%)	0.66[3]
Tumor maximal diameter in mm, median (min - max)	35 (15 – 60)	30 (10 – 90)	33 (10 – 90)	0.31[1]
Tumor stage T1 T2 T3 T4	0 (0%) 3 (10%) 25 (83%) 2 (7%)	2 (6%) 6 (17%) 23 (64%) 5 (14%)	2 (3%) 9 (14%) 48 (73%) 7 (11%)	0.33[3]
Nodal stage N1 N2	16 (53%) 14 (47%)	26 (72%) 10 (28%)	42 (64%) 24 (36%)	0.11[2]
Mucinous differentiation	2 (7%)	4 (11%)	6 (9%)	0.68[3]
Ulceration	24 (80%)	31 (86%)	55 (83%)	0.51[2]
Angio-invasion	12 (40%)	12 (33%)	24 (36%)	0.58[2]
Emergency surgery	6 (20%)	3 (8%)	9 (14%)	0.28[3]
Perforation (pre-/per-/post-operative)	2 (7%)	1 (3%)	3 (5%)	0.59[3]
Tumor spill	1 (3%)	1 (3%)	2 (3%)	1.00[3]
Disease recurrence	18 (60%)	13 (36%)	31 (47%)	0.05[2]
Local disease recurrence	3 (10%)	2 (6%)	5 (3%)	0.65[3]
Distant disease recurrence	17 (57%)	12 (33%)	29 (44%)	0.06[2]
CRC-related mortality	14 (47%)	11 (31%)	25 (38%)	0.18[2]
Overall mortality	17 (57%)	15 (42%)	32 (49%)	0.23[2]
Follow-up time in months, median (min – max)	45.1 (3.4 – 127.0)	57.2 (4.1 – 127.4)	53.0 (3.4 – 127.4)	0.19[1]

[1]Kruskal-Wallis rank sum test
[2]Pearson's Chi-squared test
[3]Fisher's exact test

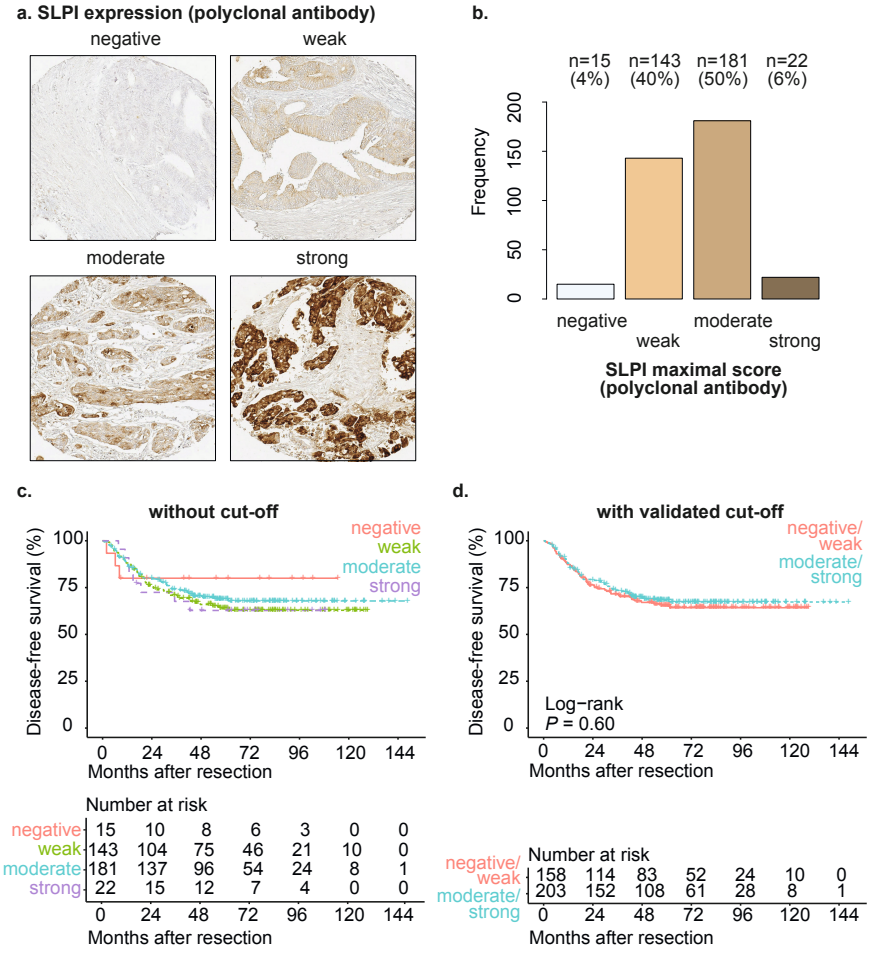
Supplementary figure 2: Clinicopathological characteristics of CRC patients included in the analysis of SLPI expression.

Patients were classified as 'SLPI-low' or 'SLPI-high' based on the validated cut-offs. Characteristics of the patients included in the analysis of SLPI expression stained with the monoclonal antibody in the whole cohort (a), in stage II CRC patients (b), in stage III CRC patients (c), in stage III MSS CRC patients (d) and in stage III MSS CRC patients treated with adjuvant chemotherapy (e).



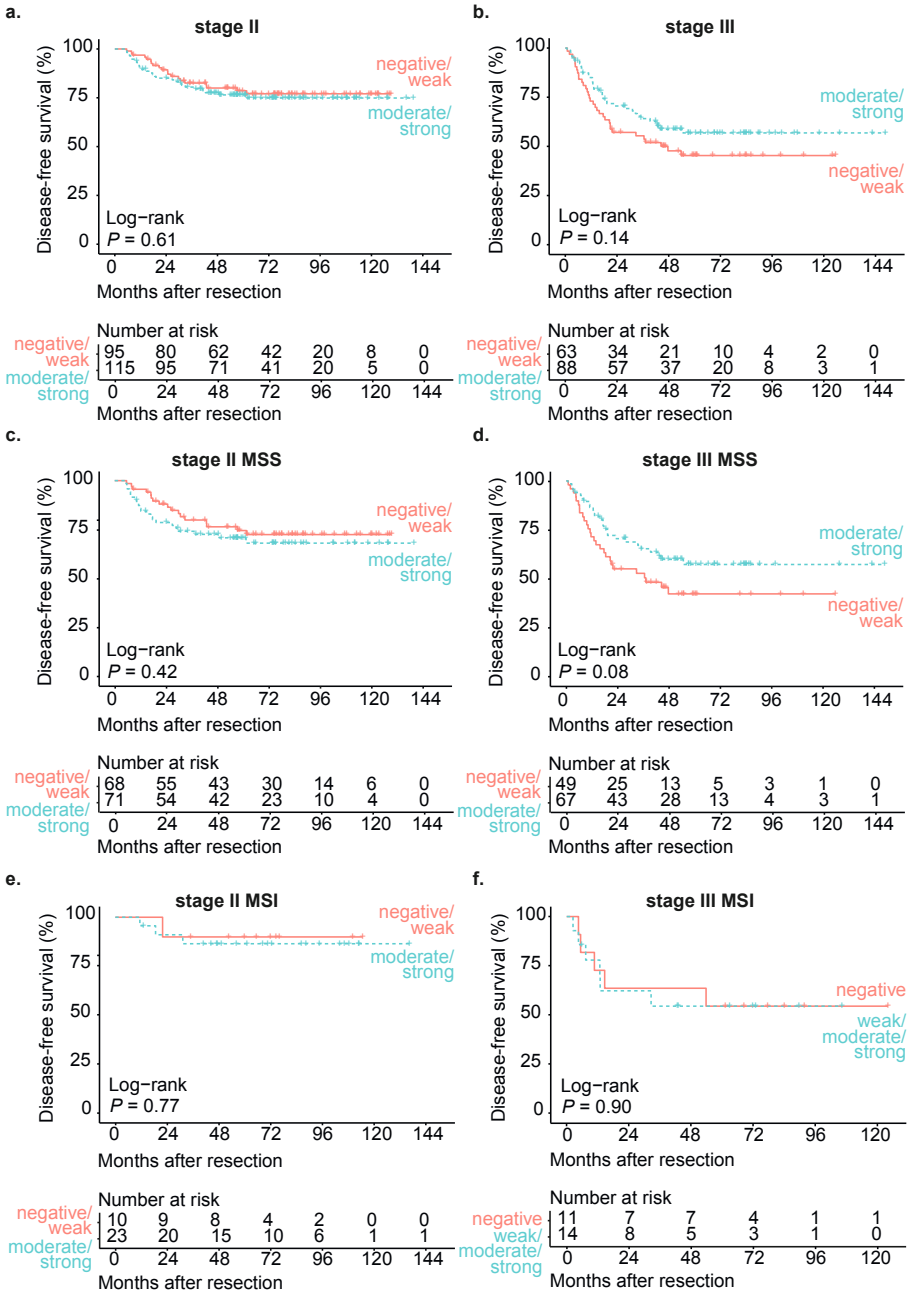
Supplementary figure 3: Relationship between SLPI detected using the monoclonal antibody and SLPI detected using the polyclonal antibody.

SLPI expression was scored for both the monoclonal antibody staining and the polyclonal antibody staining in CRC tissues from 349 stage II or stage III patients. Green fields represent the cases in which dichotomization into the 'SLPI-low' or 'SLPI-high' group based on the validated cut-offs was not different for tissues stained using the monoclonal or polyclonal antibody (77% of patients). Dotted lines represent the cut-offs for the monoclonal and polyclonal antibody. The size of the circles represents the number of patients per group, which is also indicated in the circles.



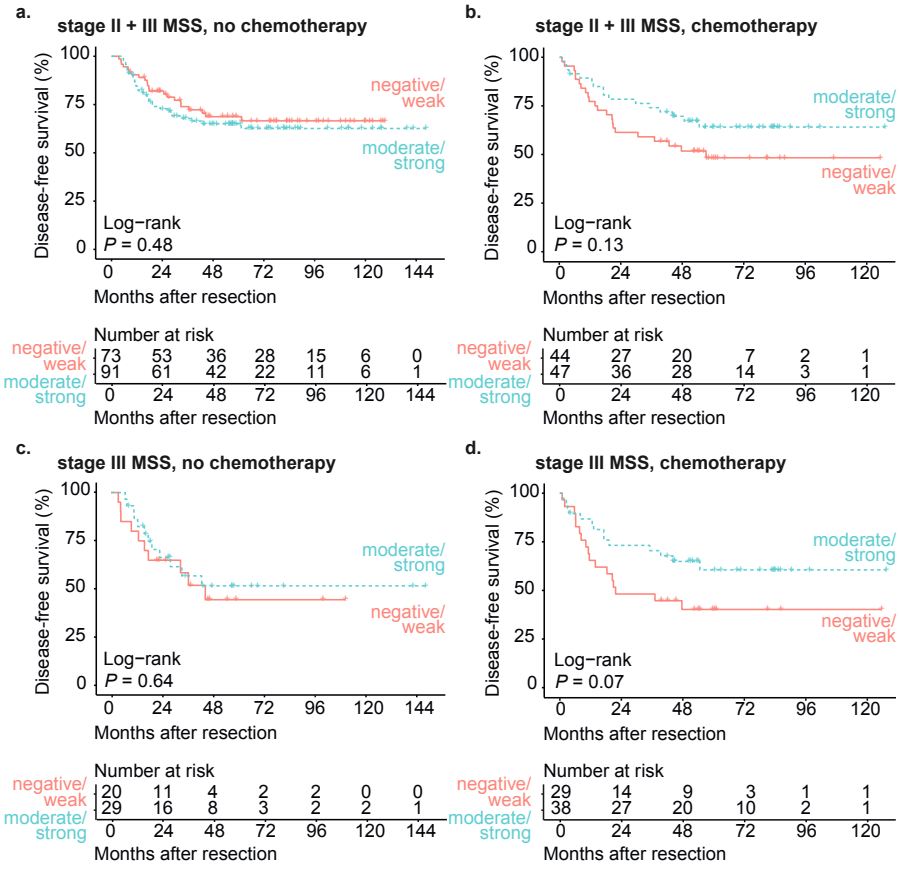
Supplementary figure 4: SLPI expression detected with the polyclonal antibody in stage II and stage III CRC.

Examples of TMA cores of stage II or stage III CRC stained for SLPI using the polyclonal antibody (a). Frequencies and percentages of stage II or stage III CRC scored as 'negative', 'weak', 'moderate' or 'strong' after staining with the polyclonal SLPI antibody (b); only the maximal score for each patient was included. Kaplan-Meier curves for disease-free survival after resection of the primary tumor (in months) for the total study population of stage II and stage III CRC patients stratified by SLPI expression detected using the polyclonal antibody (c + d, HRR 0.91, *P*-value 0.60, 95% confidence interval 0.63 – 1.31). Curves without a cut-off (c) and with the validated cut-off (d) are shown. *P*-values were calculated using the log-rank test.



Supplementary figure 5: SLPI expression detected with the polyclonal antibody in MSS and MSI CRC.

Kaplan-Meier curves for disease-free survival after resection of the primary tumor (in months) for either stage II CRC patients (a, HRR 1.16, *P*-value 0.61, 95% confidence interval 0.65 – 2.08) or stage III CRC patients (b, HRR 0.70, *P*-value 0.14, 95% confidence interval 0.44 – 1.13) stratified by SLPI expression detected using the polyclonal antibody. Kaplan-Meier curves for disease-free survival after resection of the primary tumor (in months) for stage II CRC patients with MSS (c, HRR 1.30, *P*-value 0.42, 95% confidence interval 0.69 – 2.47) or MSI tumors (e, HRR 1.39, *P*-value 0.77, 95% confidence interval 0.15 – 13.42) and stage III CRC patients with MSS (d, HRR 0.62, *P*-value 0.08, 95% confidence interval 0.36 – 1.06) or MSI tumors (f, HRR 1.08, *P*-value 0.90, 95% confidence interval 0.39 – 3.54) stratified by SLPI expression detected using the polyclonal antibody. Curves with the validated cut-off are shown. *P*-values were calculated using the log-rank test. MSS = micro-satellite stable. MSI = micro-satellite instable.



Supplementary figure 6: SLPI expression detected with the polyclonal antibody in MSS CRC patients treated with adjuvant chemotherapy.

Kaplan-Meier curves for disease-free survival after resection of the primary tumor (in months) for stage II and stage III CRC patients with MSS tumors not treated with adjuvant chemotherapy (a, HRR 1.22, *P*-value 0.48, 95% confidence interval 0.70 – 2.10) or treated with adjuvant chemotherapy (b, HRR 1.63, *P*-value 0.13, 95% confidence interval 0.32 – 1.17) stratified by SLPI expression detected using the polyclonal antibody. Kaplan-Meier curves for disease-free survival after resection of the primary tumor (in months) for stage III CRC patients with MSS tumors not treated with adjuvant chemotherapy (c, HRR 0.82, *P*-value 0.64, 95% confidence interval 0.35 – 1.89) or treated with adjuvant chemotherapy (d, HRR 0.53, *P*-value 0.07, 95% confidence interval 0.26 – 1.07) stratified by SLPI expression detected using the polyclonal antibody. Curves with the validated cut-off are shown. *P*-values were calculated using the log-rank test. MSS = microsatellite stable.

a. Characteristics of stage II and stage III patients included in the analysis of SLPI expression using the polyclonal antibody

Clinicopathological variable	SLPI-low (n=158)	SLPI-high (n=203)	Total (n=361)	*P*-value
Age in years, median (min - max)	72 (28 – 92)	73 (36 – 93)	73 (28 – 93)	0.27[1]
Gender female male	76 (48%) 82 (52%)	94 (46%) 109 (54%)	170 (47%) 191 (53%)	0.74[2]
Tumor location right (cecum until flexura lienalis) left (flexura lienalis to rectum)	71 (45%) 87 (55%)	90 (44%) 113 (56%)	161 (45%) 200 (55%)	0.91[2]
Tumor histological grade well-differentiated moderately differentiated poorly differentiated	7 (4%) 130 (82%) 21 (4%)	16 (8%) 156 (77%) 31 (15%)	23 (6%) 286 (79%) 52 (14%)	0.33[2]
Tumor maximal diameter in mm, median (min - max)	38 (10 - 130)	40 (10 – 100)	40 (10, 130)	0.99[1]
Stage II III	95 (60%) 63 (40%)	115 (57%) 88 (43%)	210 (58%) 151 (42%)	0.51[2]
Tumor stage T1 T2 T3 T4	1 (1%) 4 (3%) 136 (86%) 17 (11%)	3 (2%) 15 (7%) 166 (82%) 19 (9%)	4 (1%) 19 (5%) 302 (84%) 36 (10%)	0.18[3]
Nodal stage (Stage III patients only) N1 N2	42 (67%) 21 (33%)	63 (72%) 25 (28%)	105 (70%) 46 (31%)	0.52[2]
Mucinous differentiation	25 (16%)	49 (24%)	74 (21%)	0.05[2]
MSI-status MSI MSS Unknown	21 (13%) 117 (74%) 20 (13%)	37 (18%) 138 (68%) 28 (14%)	58 (16%) 255 (71%) 48 (13%)	0.39[2]
Ulceration	124 (79%)	151 (74%)	275 (76%)	0.37[2]
Angio-invasion	31 (20%)	36 (18%)	67 (19%)	0.65[2]
Emergency surgery	23 (15%)	26 (13%)	49 (13.6%)	0.63[2]
Perforation (pre-/per-/post-operative)	16 (10%)	15 (7%)	31 (9%)	0.36[2]
Tumor spill	3 (2%)	9 (4%)	12 (3%)	0.18[2]
Adjuvant chemotherapy	56 (35%)	63 (31%)	119 (33%)	0.38[2]
Disease recurrence	53 (34%)	62 (31%)	115 (32%)	0.54[2]
Local disease recurrence	18 (11%)	21 (10%)	39 (11%)	0.75[2]

a. (Continued)

Clinicopathological variable	SLPI-low (n=158)	SLPI-high (n=203)	Total (n=361)	*P*-value
Distant disease recurrence	43 (27%)	51 (25%)	94 (26%)	0.65[2]
CRC-related mortality	40 (25%)	52 (26%)	92 (26%)	0.95[2]
Overall mortality	64 (41%)	100 (49%)	164 (45%)	0.10[2]
Follow-up time in months, median (min – max)	60.1 (4.3 – 129.2)	57.1 (3.4 – 148.6)	57.4 (3.4 – 148.6)	0.50[1]

[1]Kruskal-Wallis rank sum test
[2]Pearson's Chi-squared test
[3]Fisher's exact test

b. Characteristics of stage II patients included in the analysis of SLPI expression using the polyclonal antibody

Clinicopathological variable	SLPI-low (n=95)	SLPI-high (n=115)	Total (n=210)	*P*-value
Age in years, median (min - max)	73 (28 – 92)	73 (40 – 92)	73 (28 – 92)	0.29[1]
Gender female male	54 (47%) 61 (53%)	50 (53%) 45 (47%)	104 (50%) 106 (51%)	0.41[2]
Tumor location right (cecum until flexura lienalis) left (flexura lienalis to rectum)	38 (40%) 57 (60%)	53 (46%) 62 (54%)	91 (43%) 119 (57%)	0.38[2]
Tumor histological grade well-differentiated moderately differentiated poorly differentiated	6 (6%) 80 (84%) 9 (10%)	11 (10%) 90 (78%) 14 (12%)	17 (8%) 170 (81%) 23 (11%)	0.54[2]
Tumor maximal diameter in mm, median (min - max)	35 (12 – 130)	40 (10 -100)	40 (10 – 130)	0.61[1]
Tumor stage T3 T4	84 (88%) 11 (12%)	103 (90%) 12 (10%)	187 (89%) 23 (11%)	0.79[2]
Mucinous differentiation	15 (16%)	29 (25%)	44 (21%)	0.10[2]
MSI-status MSI MSS Unknown	10 (11%) 68 (72%) 17 (18%)	23 (20%) 71 (62%) 21 (18%)	33 (16%) 139 (66%) 38 (18%)	0.05[2]
Ulceration	69 (73%)	87 (76%)	156 (74%)	0.62[2]
Angio-invasion	12 (13%)	8 (7%)	20 (10%)	0.16[2]
Emergency surgery	11 (12%)	14 (12%)	25 (12%)	0.90[2]

b. (Continued)

Clinicopathological variable	SLPI-low (n=95)	SLPI-high (n=115)	Total (n=210)	*P*-value
Perforation (pre-/per-/post-operative)	9 (10%)	11 (10%)	20 (10%)	0.98[2]
Tumor spill	2 (2%)	4 (4%)	6 (3%)	0.70[3]
Adjuvant chemotherapy	18 (19%)	15 (13%)	33 (16%)	0.24[2]
Disease recurrence	20 (21%)	27 (24%)	47 (22%)	0.68[2]
Local disease recurrence	9 (10%)	11 (10%)	20 (10%)	0.98[2]
Distant disease recurrence	15 (16%)	22 (19%)	37 (18%)	0.53[2]
CRC-related mortality	13 (14%)	22 (19%)	35 (17%)	0.29[2]
Overall mortality	31 (33%)	52 (45%)	83 (40%)	0.06[2]
Follow-up time in months, median (min – max)	71.9 (8.9 – 129.2)	59.0 (5.3 – 139.6)	64.3 (5.3 – 139.6)	0.20[1]

[1]Kruskal-Wallis rank sum test
[2]Pearson's Chi-squared test
[3]Fisher's exact test

c. Characteristics of stage III patients included in the analysis of SLPI expression using the polyclonal antibody

Clinicopathological variable	SLPI-low (n=63)	SLPI-high (n=88)	Total (n=151)	*P*-value
Age in years, median (min - max)	71 (34 – 87)	73 (36 – 93)	72 (34 – 93)	0.64[1]
Gender female male	26 (41%) 37 (59%)	40 (46%) 48 (55%)	66 (44%) 85 (56%)	0.61[2]
Tumor location right (cecum until flexura lienalis) left (flexura lienalis to rectum)	33 (52%) 30 (48%)	37 (42%) 51 (58%)	70 (46%) 81 (54%)	0.21[2]
Tumor histological grade well-differentiated moderately differentiated poorly differentiated	1 (2%) 50 (79%) 12 (19%)	5 (6%) 66 (75%) 17 (19%)	6 (4%) 116 (77%) 29 (19%)	0.58[3]
Tumor maximal diameter in mm, median (min - max)	40 (10 – 70)	35 (12 – 100)	35 (10 – 100)	0.63[1]
Tumor stage T1 T2 T3 T4	1 (2%) 4 (6%) 52 (83%) 6 (10%)	3 (3%) 15 (17%) 63 (72%) 7 (8%)	4 (3%) 19 (13%) 115 (76%) 13 (9%)	0.21[3]

c. (Continued)

Clinicopathological variable	SLPI-low (n=63)	SLPI-high (n=88)	Total (n=151)	*P*-value
Nodal stage N1 N2	42 (67%) 21 (33%)	63 (72%) 25 (28%)	105 (70%) 46 (31%)	0.52[2]
Mucinous differentiation	10 (16%)	20 (23%)	30 (20%)	0.30[2]
MSI-status MSI MSS Unknown	11 (18%) 49 (78%) 3 (5%)	14 (16%) 67 (76%) 7 (8%)	25 (17%) 116 (77%) 10 (7%)	0.87[2]
Ulceration	55 (87%)	64 (73%)	119 (80%)	**0.03[2]**
Angio-invasion	19 (30%)	28 (32%)	47 (31%)	0.83[2]
Emergency surgery	12 (19%)	12 (14%)	24 (16%)	0.37[2]
Perforation (pre-/per-/post-operative)	7 (11%)	4 (5%)	11 (7%)	0.20[3]
Tumor spill	1 (2%)	5 (6%)	6 (4%)	0.40[3]
Adjuvant chemotherapy	38 (60%)	48 (55%)	86 (57%)	0.48[2]
Disease recurrence	33 (52%)	35 (40%)	68 (45%)	0.13[2]
Local disease recurrence	9 (14%)	10 (11%)	19 (13%)	0.59[2]
Distant disease recurrence	28 (44%)	29 (33%)	57 (38%)	0.15[2]
CRC-related mortality	27 (43%)	30 (34%)	57 (38%)	0.27[2]
Overall mortality	33 (52%)	48 (55%)	81 (54%)	0.79[2]
Follow-up time in months, median (min – max)	46.4 (4.3 – 127.0)	51.5 (3.4 – 148.6)	50.4 (3.4 – 148.6)	0.62[1]

[1]Kruskal-Wallis rank sum test
[2]Pearson's Chi-squared test
[3]Fisher's exact test

d. Characteristics of stage III patients with MSS tumors included in the analysis of SLPI expression using the polyclonal antibody

Clinicopathological variable	SLPI-low (n=49)	SLPI-high (n=67)	Total (n=116)	*P*-value
Age in years, median (min - max)	72 (37 – 87)	72 (36 – 91)	72 (36 – 91)	0.91[1]
Gender female male	18 (37%) 31 (63%)	29 (43%) 38 (57%)	47 (41%) 69 (60%)	0.48[2]
Tumor location right (cecum until flexura lienalis) left (flexura lienalis to rectum)	22 (45%) 27 (55%)	27 (40%) 40 (60%)	49 (42%) 67 (58%)	0.62[2]
Tumor histological grade well-differentiated moderately differentiated poorly differentiated	1 (2%) 40 (82%) 8 (16%)	5 (8%) 52 (78%) 10 (15%)	6 (5%) 92 (79%) 18 (16%)	0.58[3]
Tumor maximal diameter in mm, median (min - max)	40 (10 – 70)	30 (12 – 100)	33 (10 – 100)	0.24[1]
Tumor stage T1 T2 T3 T4	1 (2%) 4 (8%) 39 (80%) 5 (10%)	3 (5%) 13 (19%) 46 (69%) 5 (8%)	4 (3%) 17 (15%) 85 (73%) 10 (9%)	0.31[3]
Nodal stage N1 N2	32 (65%) 17 (35%)	48 (72%) 19 (28%)	80 (69%) 36 (31%)	0.47[2]
Mucinous differentiation	5 (10%)	15 (22%)	20 (17%)	0.09[2]
Ulceration	42 (86%)	48 (72%)	90 (78%)	0.07[2]
Angio-invasion	15 (31%)	23 (34%)	38 (33%)	0.67[2]
Emergency surgery	10 (20%)	9 (13%)	19 (16%)	0.32[2]
Perforation (pre-/per-/post-operative)	7 (14%)	3 (5%)	10 (9%)	0.09[2]
Tumor spill	1 (2%)	4 (6%)	5 (4%)	0.40[2]
Adjuvant chemotherapy	29 (59%)	38 (57%)	67 (58%)	0.79[2]
Disease recurrence	27 (55%)	26 (39%)	53 (46%)	0.08[2]
Local disease recurrence	6 (12%)	9 (13%)	15 (13%)	0.85[2]
Distant disease recurrence	24 (49%)	20 (30%)	44 (38%)	**0.04[2]**
CRC-related mortality	23 (47%)	23 (34%)	46 (40%)	0.17[2]
Overall mortality	29 (59%)	39 (58%)	68 (59%)	0.92[2]
Follow-up time in months, median (min – max)	45.3 (4.3 – 127.0)	51.9 (3.4 – 148.6)	46.9 (3.4 – 148.6)	0.36[1]

[1]Kruskal-Wallis rank sum test
[2]Pearson's Chi-squared test
[3]Fisher's exact test

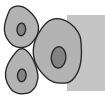
e. Characteristics of stage III patients with MSS tumors who received adjuvant chemotherapy and were included in the analysis of SLPI expression using the polyclonal antibody

Clinicopathological variable	SLPI-low (n=29)	SLPI-high (n=38)	Total (n=67)	*P*-value
Age in years, median (min - max)	66 (37 – 83)	67 (36 – 82)	66 (36 – 83)	0.80[1]
Gender female male	10 (35%) 19 (66%)	12 (32%) 26 (68%)	22 (33%) 45 (67%)	0.80[2]
Tumor location right (cecum until flexura lienalis) left (flexura lienalis to rectum)	15 (52%) 14 (48%)	15 (40%) 23 (61%)	30 (45%) 37 (55%)	0.32[2]
Tumor histological grade well-differentiated moderately differentiated poorly differentiated	0 (0%) 25 (86%) 4 (14%)	4 (11%) 27 (71%) 7 (18%)	4 (6%) 52 (78%) 11 (16%)	0.18[3]
Tumor maximal diameter in mm, median (min - max)	35 (10 – 60)	30 (12 – 60)	30 (10 – 60)	0.40[1]
Tumor stage T1 T2 T3 T4	0 (0%) 2 (7%) 24 (83%) 3 (10%)	2 (5%) 7 (18%) 24 (63%) 5 (13%)	2 (3%) 9 (13%) 48 (72%) 8 (12%)	0.30[3]
Nodal stage N1 N2	17 (59%) 12 (41%)	25 (86%) 13 (34%)	42 (63%) 25 (37%)	0.55[2]
Mucinous differentiation	2 (7%)	4 (11%)	6 (9%)	0.69[3]
Ulceration	24 (83%)	30 (79%)	54 (81%)	0.70[2]
Angio-invasion	10 (35%)	14 (37%)	24 (36%)	0.84[2]
Emergency surgery	6 (21%)	4 (11%)	10 (15%)	0.31[3]
Perforation (pre-/per-/post-operative)	3 (10%)	0 (0%)	3 (5%)	0.08[3]
Tumor spill	1 (3%)	1 (3%)	2 (3%)	1.00[3]
Disease recurrence	17 (59%)	14 (37%)	31 (46%)	0.09[3]
Local disease recurrence	3 (10%)	3 (8%)	6 (9%)	1.00[3]
Distant disease recurrence	17 (59%)	11 (29%)	28 (42%)	**0.02[2]**
CRC-related mortality	14 (48%)	12 (32%)	26 (39%)	0.17[2]
Overall mortality	16 (55%)	17 (45%)	33 (49%)	0.40[2]
Follow-up time in months, median (min – max)	46.4 (4.3 – 127.0)	56.0 (3.4 – 127.4)	52.5 (3.4 – 127.4)	0.25[1]

[1]Kruskal-Wallis rank sum test
[2]Pearson's Chi-squared test
[3]Fisher's exact test

Supplementary figure 7: Clinicopathological characteristics of CRC patients included in the analysis of SLPI expression.

Patients were classified as 'SLPI-low' or 'SLPI-high' based on the validated cut-offs. Characteristics of the patients included in the analysis of SLPI expression stained with the polyclonal antibody in the whole cohort (a), in stage II CRC patients (b), in stage III CRC patients (c), in stage III MSS CRC patients (d) and in stage III MSS CRC patients treated with adjuvant chemotherapy (e).



CHAPTER 7

Discussion and conclusions

The main aims of this thesis were to elucidate whether Secretory Leukocyte Protease Inhibitor (SLPI) expression in the intestine of pediatric inflammatory bowel disease (IBD) patients is associated with an immune subtype of disease and whether SLPI expression in colorectal cancer (CRC) predicts patient prognosis. In addition, we aimed to identify the role of SLPI in the healthy intestine, in the pathogenesis of IBD, and in CRC progression.

1. SLPI IN INTESTINAL HOMEOSTASIS

1.1 Which cells in the intestine express SLPI?

Our data demonstrate that SLPI is expressed by intestinal epithelial cells both during homeostasis and inflammation. We detected SLPI protein expression in intestinal epithelial cells of both children with IBD and in a control group of children suspected of having IBD but who received a negative diagnosis based on endoscopic and histopathological evaluation [chapter 3]. Others have detected SLPI in epithelial cells at the basis of crypts and in goblet cells scattered throughout the epithelium in the healthy colon (1). We did not unequivocally establish which intestinal epithelial cells express SLPI, but our immunohistochemical analysis and RNA sequencing data argue that SLPI is mainly produced by goblet cells [chapter 3]. In line with this, we observed that both epithelial *SLPI* mRNA and SLPI protein expression are significantly higher in the colon compared to the small intestine [chapter 3]. We previously observed the same pattern in the murine intestinal epithelium (2). There are multiple possible explanations for this observation. First, the number of goblet cells is higher in the colon compared to the small intestine. If these cells are indeed the main producers of SLPI, fewer SLPI producing cells are present in the small intestine and production of other antimicrobial proteins by other epithelial cells such as Paneth cells may be more important in the small intestine. Secondly, the higher number of bacteria in the colon compared to the small intestine may result in higher SLPI expression by colonic epithelial cells, as SLPI expression in intestinal epithelial cells is induced by Toll-like receptor (TLR) ligands (2). Alternatively, specific micro-organisms present in the colon may upregulate epithelial SLPI expression. However, *in vitro* epithelial SLPI expression can be upregulated by a wide variety of TLR ligands, suggesting that many different micro-organisms can stimulate epithelial SLPI expression (2). In addition, cytokines, growth factors and hormones are also able to increase SLPI expression in epithelial cells (3-7). Although we found that IL-17A upregulates SLPI expression in epithelial cells *in vitro* [chapter 3], we detected similar numbers of IL-17-positive cells in the human colon and small intestine [data not shown]. In addition, in mice IL-17A secreting cells are more abundant in the small intestine than in the colon (8), suggesting that differences in IL-17A production do not account for the differential SLPI expression

in these compartments. Transforming growth factor alpha (TGF-α) and Insulin-like growth factor 1 (IGF-1) both increase SLPI expression in human keratinocytes (9), but protein expression of TGF-α and IGF-1 is not different between the small intestine and the colon (Human Protein Atlas project (10)). Progesterone and corticosteroids can also increase SLPI expression in epithelial cells, but whether the concentration of these hormones is different between the small intestine and the colon is unknown (6,7).

Apart from epithelial SLPI expression, we also detected SLPI in the cytoplasm and nucleus of mononuclear cells in the human intestinal lamina propria [chapter 4]. The frequencies of these SLPI-producing mononuclear cells are low and the expression is more focal than in colonic epithelium. However, this data is consistent with the observed SLPI expression in mononuclear cells of the murine intestine (2). In the human intestinal lamina propria, we show that these SLPI-positive cells are CD68-positive and therefore are monocytes or macrophages [chapter 4]. Human alveolar macrophages have been reported to express SLPI (11). In addition, we found that human THP-1 monocytic cells also express and produce SLPI [chapter 4]. Whether the majority of SLPI-positive cells in the human intestinal lamina propria are indeed monocytes or macrophages remains to be demonstrated.

1.2 What is the function of SLPI in the healthy intestine?

The gastrointestinal immune system evolved to downregulate inflammatory responses but not antimicrobial responses to micro-organisms that breach the epithelial barrier (12). SLPI has multiple anti-inflammatory and antimicrobial functions, which may protect the healthy intestine [chapter 2].

Inhibition of NF-κB signaling

First, SLPI inhibits nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling in intestinal epithelial cells and thereby decreases chemokine and cytokine production in response to microbial signals (2). This prevents unwanted inflammatory responses to harmless commensal bacteria and inhibits subsequent tissue damage. In addition, SLPI expression by dendritic cells in mucosa-draining lymph nodes attenuates their activation in response to lipopolysaccharide (LPS) (13). This protects against inflammatory T-cell responses to the microbiota. It has been reported that SLPI inhibits TLR signaling at three levels: extracellular by interfering with the binding of LPS to CD14 (14); in the cytosol by preventing the degradation of the NF-κB inhibitor alpha (IκBα) (15); and in the nucleus by competing with p65 for NF-κB consensus-binding sites (16). In our laboratory, we have not been able to reproduce the extracellular function of SLPI yet (data not shown). In addition, the nuclear binding of SLPI to p65 is technically challenging to detect. However, we have demonstrated

that SLPI inhibits NF-κB signaling in monocytes via prevention of degradation of IκBα [chapter 4].

In order to assess the relative contribution of SLPI to inhibition of NF-κB signaling, we compared expression of SLPI in human buccal epithelial cells (2) and human monocytic cells [chapter 4] to expression of other NF-κB inhibitors and found that SLPI expression was highest. However, in murine buccal epithelial cells expression of the NF-κB inhibitor Toll-interacting protein (TOLLIP) is expressed much higher compared to SLPI and other NF-κB inhibitors, possibly suggesting that SLPI is less dominant as an NF-κB inhibitor in the murine gastrointestinal tract (2).

SLPI inhibits NF-κB signaling both in epithelial cells and in monocytic cells. In both cell types, this leads to suppression of chemokine and cytokine production, but the consequences may be different. Under homeostatic conditions, circulating monocytes are recruited to the intestine to replenish resident mucosal macrophages, which undergo apoptosis after weeks to months (12). These resident intestinal macrophages are able to phagocytose and kill micro-organisms without causing an inflammatory response, in part due to downregulation of NF-κB via multiple mechanisms (12). In contrast, during infection monocytes are recruited to the intestine as part of an antimicrobial response (12). Thus, newly recruited monocytes need to adapt to the environment to either maintain homeostasis or fight infections. We observed that endogenous SLPI production by monocytic cells results in inhibition of chemokine and cytokine production in response to microbial signals [chapter 4]. Therefore, under homeostastic conditions SLPI may prevent excessive activation of monocytes after entry into the intestinal tissue [chapter 4]. Possibly, during a breach of barrier and consecutive microbial translocation TLR ligands and cytokines upregulate SLPI in recently migrated monocytes, resulting in suppression of the inflammatory response via inhibition of NF-κB. In contrast, during infection IFN-γ may overrule SLPI-induced hyporesponsiveness to microbial signals, as has been described for murine macrophages (17), resulting in a strong pro-inflammatory response. Thus, the role of SLPI expression in monocytes during intestinal inflammation should be studied further, for example using mouse models for intestinal inflammation with conditional knockout of SLPI in monocytes.

Inhibition of proteases

It is likely that SLPI protects the intestine against tissue damage by proteases. *In vitro*, SLPI protects intestinal epithelial cells against neutrophil elastase and trypsin activity (3). *In vivo*, SLPI ameliorates dextran sodium sulphate (DSS)-colitis both in wildtype mice (18) and in thymic stromal lympopoietin (TSLP)-deficient mice, who suffer from

excessive colonic neutrophil elastase activity (19). In SLPI-deficient mice, DSS colitis is more severe compared to wildtype mice due to increased neutrophil elastase (20). In this study, administration of an inhibitor of neutrophil elastase to SLPI-deficient mice ameliorated DSS-colitis (20). However, whether neutrophil elastase activity fully explains the observed increase in colitis severity in the absence of SLPI is unclear. Thus, inhibition of proteases by SLPI protects the intestine, but in contrast to the lungs the relevance of this function in the intestine is not well established [chapter 2].

Antimicrobial activity

Finally, SLPI is known to have strong antimicrobial activity. SLPI kills both gram-negative and gram-positive bacteria at physiological concentrations, including *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis* and *Salmonella typhimurium* (3,21-24). Thus, SLPI may protect against intestinal pathogens including *Salmonella typhimurium,* and may also have a role in shaping the intestinal microbiota. However, SLPI's antimicrobial activity in the intestine is not well studied.

Conclusion

In conclusion, SLPI prevents the attraction and activation of leukocytes after microbial contact, prevents tissue damage caused by neutrophils and may kill invading pathogenic micro-organisms in the intestine. However, whether SLPI is indispensable for intestinal homeostasis remains unsolved. To date, no congenital genetic alterations in SLPI have been reported in humans. However, based on our results and the literature it is plausible that SLPI has a protective role during intestinal infection and inflammation in both humans and mice.

2. SLPI IN IBD

2.1 How is SLPI expression altered in IBD?

In the healthy intestine, SLPI expression is acquired at birth, the time when the colonic mucosal immune system first encounters microbial stimuli. Colonization of germ-free mice with benign commensal microbiota elicits a gradually increased SLPI expression that stabilizes around 30 days after colonization (2). Similarly, primary human buccal epithelial cells collected directly after birth do not yet express SLPI while primary human buccal epithelial cells from older healthy children do (2). Which mechanisms account for the increased epithelial SLPI expression in biopsies from patients with IBD compared to biopsies from IBD-negative patients [chapter 3]? As *in vitro* experiments clearly have established that TLR ligation can increase SLPI expression in epithelial cells, increased microbial-host interaction may be a strong driver of enhanced epithelial SLPI expression *in vivo*. Indeed, as demonstrated in chapter 3, epithelial barrier breach

allowing for microbial translocation is sufficient to induce high colonic SLPI expression in mucin-2 deficient mice and DSS-treated wild-type mice. However, in these *in vivo* models it is difficult to disentangle increased microbial-epithelial contact from the immune activation that takes place simultaneously. In consequence, it is possible that immune activation coincides with microbial activation, together driving increased SLPI expression. We show that IL-17A upregulates SLPI expression in buccal epithelial cells [chapter 3], suggesting that, next to microbial signals, IL-17A can increase SLPI expression in the intestinal epithelium in IBD. This is in agreement with the fact that IBD patients with high colonic SLPI expression exhibit increased frequencies of IL-17 producing cells in the same lesions and have higher plasma concentrations of Th17-associated cytokines including IL-17A [chapter 3]. It remains to be established whether IL-17 production needs to precede microbial stimulation to induce enhanced SLPI expression. This has been suggested to occur in a murine model for lung colonization. In particular, similar to the intestine, *Slpi* mRNA expression is upregulated in the lungs of germ-free mice after colonization with *Bordetella pseudohinzii* which also elicits Th17 differentiation. Intriguingly, colonization of mice that lack lymphocytes fails to elicit *Slpi* mRNA expression in the lungs, suggesting that Th17-like responses may be a prerequisite for SLPI expression (25). Whether other inflammatory cytokines, besides IL-17, contribute to increased colonic SLPI expression in IBD remains to be established. While transcription of SLPI in human alveolar epithelial cell line A549 is reported to be increased by co-stimulation of TNF-α and IL-17 but not by either cytokine alone (25), our *in vitro* experiments with the buccal epithelial cell line TR146 show that SLPI mRNA and protein expression are significantly increased by IL-17A, but not by TNF-α [chapter 3]. Moreover, IFN-γ appeared to reduce SLPI expression rather than stimulate it in TR146 cells [chapter 3]. These data suggest that SLPI may be upregulated in intestinal epithelial cells by increased microbial contact not only directly, but also indirectly via production of IL-17A by Th17 lymphocytes.

As we aimed to determine whether SLPI expression in IBD is associated with a subtype of disease, we asked how colonic epithelial SLPI expression is different between IBD patients. First, we compared SLPI in ulcerative colitis (UC) patients and Crohn's disease (CD) patients. SLPI protein expression was higher in the most affected colonic biopsy from UC patients compared to CD patients [chapter 3]. This observation may be related to the fact that the most affected colonic biopsy from CD patients was not actively inflamed for every patient due to the discontinuous and patchy nature of the disease, in contrast to UC. Indeed, we found that colonic epithelial SLPI expression is related to both the degree of macroscopic and microscopic inflammation [chapter 3]. High epithelial SLPI protein expression in the most affected colonic biopsy was associated with higher endoscopic scores, which are composed of macroscopic characteristics

[chapter 3]. These data suggest that colonic epithelial SLPI expression in IBD reflects a biological process related to severe ongoing colonic inflammation. High epithelial colonic SLPI expression was indeed associated with high microscopic disease activity as assessed by the Global Histological disease Activity Score (GHAS) [chapter 3]. The GHAS is a complex scoring method and the level of agreement among pathologists has not been established. Therefore, there is a need for one parameter which reflects both microscopic disease activity and disease severity at the patient level. We showed that SLPI expression in the colonic epithelium provides information not only about the part of the intestine where the biopsy was taken from, but also about the severity of disease in the individual patient. In particular, we found a Th17-profile in the plasma of patients with high colonic epithelial SLPI protein expression [chapter 3]. These data argue that by detecting SLPI expression in a colonic biopsy, IBD patients with high clinical disease activity at diagnosis can be differentiated from IBD patients with less clinical disease activity at diagnosis. Whether this difference in clinical disease severity is related to the phase of disease or the underlying pathogenesis remains an important question. Importantly, we showed that colonic epithelial SLPI expression in IBD is not only related to the degree of inflammation but also to a particular type of inflammation. Colonic epithelial SLPI expression is associated with strong immune activation characterized by neutrophil infiltration and IL-17A production [chapter 3]. Therefore, SLPI expression may reflect a strong antimicrobial response occurring in a subgroup of IBD patients, related to underlying innate immune defects or due to a peak of disease activity. If SLPI expression is indeed related to underlying innate immune defects, patients with high colonic SLPI expression may benefit from a different therapeutic strategy and may have a different course of disease. To assess the predictive and prognostic value of SLPI in IBD, long term follow-up of pediatric IBD patients is ongoing in our laboratory.

We found that high colonic epithelial SLPI expression is associated with a high number of neutrophils in the tissue [chapter 3]. In addition, we found a gene expression profile of strong neutrophil activation and Th17 signaling in colonic biopsies from patients with high colonic epithelial SLPI expression [chapter 3]. Neutrophils have been implicated in a complicated disease course and therapy resistance in IBD, but the exact role of neutrophils in IBD is unclear (26). We showed that high expression of SLPI is related to recruitment and activation of neutrophils. West et al. have shown that high expression of Oncostatin M (OSM), a neutrophil product, in the inflamed intestine of IBD patients is associated with nonresponse to anti-TNF therapy (27). However, the relation between OSM expression and clinical and immune disease characteristics of IBD patients were unclear. We observed that OSM and OSM-associated genes (27) were upregulated in colonic biopsies from patients with high epithelial SLPI expression

compared to patients with low SLPI expression [chapter 3]. IL-17A may both enhance SLPI expression and neutrophil recruitment in IBD, as we found that IL-17A upregulates SLPI in buccal epithelial cells *in vitro* [chapter 3] and IL-17A is a neutrophil attractant (28). Thus, our data argue that a strong antimicrobial response characterized by Th17 differentiation and high epithelial SLPI expression is related to strong recruitment and activation of neutrophils, resulting in more severe disease and possibly also therapy resistance. Whether high colonic epithelial SLPI expression indeed predicts therapy resistance will be investigated by long term follow-up of these patients in our laboratory.

Detecting SLPI by immunohistochemistry is more informative in the context of IBD compared to detecting SLPI expression in bulk RNA sequencing. Previously, SLPI has been overlooked in analyses of mRNA expression, as epithelial cells are not always abundant in intestinal biopsies. For example, a gene signature characterized by high *IL1B* expression was found to be strongly related to histological inflammation, in particular ulceration, in intestinal tissue from IBD patients nonresponsive to anti-TNF therapy and corticosteroids (29). SLPI is not part of this signature, but epithelial cells were significantly decreased in these IBD tissues (29). Therefore, using paired biopsies we compared gene expression between patients with high and low colonic epithelial SLPI protein expression as detected by immunohistochemistry [chapter 3]. By scoring the intensity of SLPI protein expression in epithelial cells independent of the number of epithelial cells, we were able to semi-quantify SLPI expression independent of the composition of the biopsy. As expected, high epithelial protein SLPI expression was significantly associated with higher *SLPI* mRNA expression, but some patients with high SLPI scores had low *SLPI* mRNA expression, most likely related to a low number of epithelial cells in the biopsy as suggested by relatively low expression of *Epithelial cell adhesion molecule (EPCAM)* mRNA [chapter 3]. Thus, by detecting SLPI protein expression using immunohistochemistry, we were able to reliably assess SLPI expression in IBD and find the association between epithelial SLPI expression and neutrophil activation and IL-17 signaling.

2.2 What is the function of SLPI in IBD?

We showed that high colonic epithelial SLPI expression identifies pediatric IBD patients with active disease and strong immune activation characterized by extensive neutrophil infiltration and IL-17A production. This led us to question whether SLPI is beneficial or detrimental during IBD. In view of SLPI's anti-inflammatory, tissue protective and antimicrobial functions we anticipated that SLPI expression is beneficial during IBD. SLPI may prevent attraction of neutrophils to the intestine, limit tissue

damage caused by neutrophils and may kill micro-organisms which have translocated to the lamina propria (Figure 1).

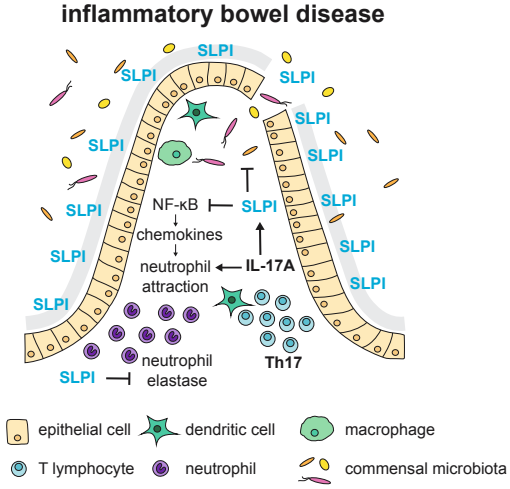


Figure 1: In inflammatory bowel disease, epithelial SLPI expression is upregulated by microbial contact and IL-17A and may limit tissue damage

In inflammatory bowel disease, barrier defects or innate immune defects lead to translocation of bacteria to the lamina propria. Increased contact between epithelial cells and the microbiota leads to upregulation of SLPI expression via TLR ligands. In addition, bacterial translocation leads to differentiation of naive T cells into Th17 lymphocytes, which produce IL-17A. IL-17A further upregulates SLPI expression in intestinal epithelial cells and attracts neutrophils. SLPI in turn inhibits chemokine production via NF-κB inhibition, thereby limiting neutrophil attraction. In addition, SLPI prevents tissue damage by inhibiting neutrophil elastase. Finally, SLPI may also kill micro-organisms which have translocated to the lamina propria.

The role of SLPI expression in monocytes in IBD is unknown. We detected CD68-positive SLPI-positive cells in the lamina propria of patients with IBD and non-IBD patients [chapter 4]. However, only a subset of CD68-positive cells was SLPI-positive [chapter 4]. As we showed that SLPI is a strong inhibitor of monocyte activation [chapter 4], our data suggest that only a subpopulation of monocytes or macrophages express SLPI and that these may be functionally distinct. In IBD, proinflammatory molecules are mainly produced by monocytes and neutrophils that have migrated to the inflammatory site (30). SLPI expression by monocytes may thus prevent unwanted activation of monocytes and subsequent cytokine and chemokine production. However, we do not know whether the number of SLPI-expressing monocytes or the expression of SLPI is increased in IBD patients. As SLPI-positive cells are not evenly dispersed in the tissue and were often clustered in small foci around destructed crypts, these cells are difficult to quantify in biopsies. Therefore, mouse models with

conditional knockout of SLPI in monocytes are necessary to study the function of SLPI in monocytes during intestinal inflammation.

Whether inhibition of NF-κB signaling by SLPI has a role in IBD is unclear. NF-κB is activated in the intestine of IBD patients, resulting in increased expression of numerous proinflammatory cytokines and chemokines (30). Inhibition of NF-κB can attenuate experimental colitis induced by 2,4,6,-trinitrobenzene sulfonic acid (TNBS), a transmural granulomatous colitis (31). In contrast, NF-κB signaling can also have beneficial effects during inflammation, such as protection of epithelial cells against apoptosis (32,33). In addition, mice deficient in myeloid differentiation protein MyD88, an adaptor molecule essential for TLR signaling which activates NF-κB, are more susceptible to DSS-colitis compared to wildtype littermates (34,35). The presumed mechanism for this finding is protection against epithelial injury caused by recognition of commensal bacteria and subsequent TLR signaling (35). Defective NF-κB signaling in intestinal epithelial cells can indeed lead to intestinal inflammation in patients (36). Thus, by inhibiting NF-κB signaling, SLPI may also inhibit protective processes, leading to epithelial injury. In IBD, apoptosis of epithelial cells may further result in barrier defects, aggravating bacterial translocation. It is therefore possible that upregulation of SLPI during IBD is not only protective.

Another question that remains is whether inhibition of NF-κB signaling by SLPI in epithelial cells or monocytes could also lead to insufficient innate immune responses. We aimed to address this question by studying SLPI knockout mice during DSS colitis. However, we did not find clear differences between SLPI knockout mice and littermates during DSS-colitis (data not shown), in contrast to others (20). Investigating SLPI function by studying SLPI knockouts is complex for multiple reasons. First, SLPI has diverse functions, which makes it difficult to unravel which function is crucial in each model. Secondly, it is possible that other NF-κB inhibitors compensate for SLPI's function in SLPI knockout mice and therefore an effect on NF-κB signalling may be limited. Finally, SLPI expression is microbiota-induced and its functions are most relevant in the presence of microbiota. Mice in animal facilites are kept under specific pathogen-free (SPF) conditions and may therefore not be suitable to study SLPI's function. As discussed earlier, SLPI knockout mice do not develop spontaneous intestinal inflammation under SPF conditions (37). However, in the presence of micro-organisms that trigger inflammation, SLPI may be essential to prevent tissue damage. We detected increased SLPI expression in the colon of wildtype mice during DSS-colitis under SPF conditions, demonstrating that increased contact with the SPF microbiota leads to upregulation of SLPI [chapter 3]. However, to find phenotypic differences

between SLPI knockout mice and wildtype littermates during DSS-colitis, colonization with micro-organisms that cause a stronger inflammatory response may be required.

Whether administration of recombinant SLPI in the intestine or stimulation of endogenous SLPI production could ameliorate IBD is an interesting question. However, the literature discussed above warrants for mechanistic studies investigating the effect of SLPI on intestinal epithelial cells. In addition, as discussed in the next paragraph, SLPI may have a role during carcinogenesis which needs to be elucidated before SLPI should be administered in patients.

3. SLPI IN COLORECTAL CANCER

3.1 How is SLPI expression altered in colorectal cancer cells?

SLPI is upregulated in several types of cancer (38-43). We found that SLPI protein expression is high in a subset of CRC liver metastases and matching primary tumors from CRC patients after resection of liver metastases [chapter 5]. In addition, we found high SLPI protein expression in the tumors of a subgroup of stage II patients (patients without metastases) and stage III patients (patients with lymph node metastases but no distant metastases). Thus, in a subset of tumors, SLPI expression may already be upregulated before metastasis occurs. The mechanism by which SLPI expression is upregulated in some CRC tumors is unknown. Possibly, SLPI expression is regulated by cytokines in the tumor micro-environment. Alternatively, contact between tumor cells and micro-organisms could result in upregulation of SLPI. It is also possible that SLPI is overexpressed due to genetic or epigenetic alterations, but this has not been reported. Interestingly, we found an association between SLPI protein expression and mucinous differentiation [chapter 6], suggesting that SLPI expression may be related to tumor cell differentiation.

Survival of stage II and stage III CRC patients after resection of the primary tumor is highly variable and cannot be predicted well based on currently known clinical and pathological risk factors (44,45). In addition, survival is also highly variable in patients with CRC liver metastases after resection of liver metastases with curative intention. Therefore, for both patient groups there is a need for prognostic factors to select patients which will benefit from additional therapy. In CRC patients with liver metastases, we found that SLPI protein expression is associated with shorter overall survival independently of established clinical risk factors [chapter 5]. Thus, our data suggest that detection of SLPI expression using immunohistochemistry may aid in prediction of prognosis in patients with CRC liver metastases. In stage II CRC patients, we found that tumor SLPI protein expression did not have prognostic value. In contrast,

high tumor SLPI protein expression was associated with reduced disease recurrence in stage III micro-satellite stable (MSS) CRC patients treated with adjuvant Fluorouracil (5-FU)-based chemotherapy, independently of established clinical risk factors [chapter 6]. Remarkably, we did not observe this association in stage III CRC patients with micro-satellite instable (MSI) tumors. The reason for this observation remains unclear. MSI tumors are known to elicit a stronger anti-tumor immune response compared to MSS tumors (46). Therefore, we expected higher SLPI expression in MSI-tumors, but SLPI expression was not different between MSI and MSS-tumors [chapter 6], suggesting that SLPI expression in CRC is influenced by other factors than cytokines in the tumor microenvironment alone.

3.2 What is the function of SLPI in colorectal cancer?

The function of SLPI in CRC tumor initiation, tumor growth and metastasis formation is unknown. The prognostic value of SLPI in CRC patients with liver metastases suggests a role for SLPI in metastasis formation in human CRC [chapter 5]. However, SLPI may have different functions during the different stages of disease, as we found that SLPI expression in stage III MSS CRC is associated with better prognosis [chapter 6], whereas in CRC patients with liver metastases SLPI expression is associated with poorer prognosis [chapter 5]. As the prognosis of CRC patients is largely determined by the development of metastases, our data suggest that high SLPI expression may reflect a metastasis promoting process or has a pro-metastatic effect at the stage at which metastases have already formed. In contrast, in the stage at which lymph node metastases but not distant metastases have formed, high SLPI expression may counteract the development of distant metastases. Alternatively, SLPI may make tumors more susceptible to chemotherapy. It is not surprising that the relation between SLPI expression and CRC patient prognosis is complex, as SLPI may both counteract and promote cancer via multiple mechanisms [chapter 2]. Most studies indicate that SLPI promotes metastasis formation (39,47), but SLPI may also inhibit tumor initiation and tumor growth (48-50). However, mechanistic analyses are needed to elucidate the function of SLPI in the different stages of CRC.

Chronic inflammation increases the risk of many cancers, including CRC (51). The gastrointestinal tract is highly susceptible to chronic inflammation with consequent tumor development (52). Patients with IBD patients have an increased risk of developing CRC, but the exact mechanism is not entirely clear (53). Chronic inflammation can cause genomic changes and the interplay between the immune system and cancer is highly complex, as both tumor promoting inflammation and anti-tumor immune responses can co-exist during carcinogenesis (51). The expression of immune modulators in the tumor microenvironment determines the balance between

tumor-promoting inflammation and anti-tumor immunity (51). Importantly, the net effect of inflammation is most often stimulation of tumor growth (52). In particular, innate immune cells have an important tumor-promoting role by contributing to multiple cancer hallmark capabilities via the production of molecules including growth factors, survival factors, proangiogenic factors, chemokines and cytokines (54). On the other hand, lymphocytes and innate immune cells can also mount relevant tumor-killing responses (54).

NF-κB is a key link between chronic inflammation and cancer, as it is a crucial mediator of tumor growth (52). NF-κB activation is triggered by various cytokines including TNF-α and IL-1β, and by pathogen-associated molecular patterns. However, the effect of NF-κB signaling on the initiation of tumors and tumor growth is different per cell type (52). The role of NF-κB activation in intestinal epithelial cells has been investigated using a mouse model of colitis-associated cancer (52). In this model, injection with azoxymethane (AOM), a procarcinogen, followed by multiple cycles of DSS administration in the drinking water induces tumors in the distal colon. Using this model, decreased NF-κB activity in enterocytes was found to decrease tumor incidence via increased apoptosis, without affecting the induction of oncogenic mutations, tumor size or tumor progression (52). In contrast, decreased NF-κB activity in myeloid cells resulted in a decrease in the incidence of tumors and a decrease in tumor size, with a more pronounced effect on tumor size, due to decreased production of growth factors by myeloid cells (52). These growth factors, including IL-1, IL-6 and TNF-α, are necessary for the proliferation of neoplastic epithelial cells (52). Thus, inhibition of intestinal epithelial NF-κB by SLPI may prevent early tumor growth via increased apoptosis. In addition, inhibition of NF-κB in myeloid cells by SLPI may suppress tumor growth via inhibition of the production of tumor-promoting cytokines and growth factors.

Possibly, chronic intestinal inflammation may also contribute to CRC initiation by inducing epithelial dedifferentiation. Elevated NF-κB signaling can induce dedifferentiation of intestinal epithelial cells via Wnt activation, resulting in the reacquisition of stem cell properties (55). Via this mechanism, chronic intestinal inflammation as occurs in IBD may result in an increase in the number of potentially tumor-initiating cells (55). Therefore, inhibition of NF-κB signaling by SLPI may prevent intestinal epithelial carcinogenesis. Thus, inhibition of NF-κB signaling in tumor development can lead to diverse outcomes and therefore the net effect of increased SLPI expression by tumor cells is difficult to predict. We aimed to identify the role of SLPI in colitis-associated cancer by comparing tumor development between SLPI deficient mice and wildtype littermates in the AOM/DSS model. Both wildtype and

SLPI-deficient mice developed colorectal tumors, but we did not have sufficient power to draw definite conclusions (data not shown). In addition, the tumors in the AOM/DSS model are mostly adenomas and not carcinomas (44) and therefore this model is not suitable to study invasion of cancer cells or metastasis formation.

We hypothesized that SLPI prevents the recruitment of immune cells to the tumor niche, as it suppresses chemokine production by intestinal epithelial cells. This could lead to suppressed tumor growth production, but also to less anti-cancer immunity. Indeed, in colitis-associated CRC, infiltrating $CD8^+$ T cells may promote cancer by contributing to intestinal inflammation (56). A possible role for SLPI in immune evasion by cancer cells has not been examined yet. Interestingly, in a study on heritable non-genetic mechanisms of cancer evolution, dominant clones in a mouse model for acute myeloid leukemia have high expression of *Slpi* in common (57). Reduced *Slpi* expression impaired clonal fitness in this model and the top differentially expressed genes in clones that maintained dominance after transplantation into a secondary mouse recipient were increased expression of *Slpi* and decreased expression of *Beta-2-microglobulin*, enconding a component of the major histocompatibility (MHC) system (57). It was therefore suggested that immune evasion of cancer cells may be related to clonal fitness (58). However, the exact mechanism by which SLPI may promote malignant clonal fitness was not examined.

SLPI has more functions besides inhibition of NF-κB which may also counteract or contribute to tumor initiation, tumor growth and metastasis. In a mouse model for breast cancer, clones expressing *Slpi* more efficiently enter the vasculature, suggesting that SLPI has a cancer-promoting role at the initial stage of metastasis (39). However, this does not explain why we observed a relationship between high SLPI expression and poor prognosis only in stage IV and not in stage II or stage III CRC patients. In addition, in the same mouse model SLPI's anticoagulant function also contributed to metastasis formation (39), but whether SLPI promotes CRC metastasis via anticoagulation remains to be investigated. In contrast, inhibition of proteases by SLPI may inhibit cancer cell invasion as proteolysis is required for the invasion of cancer cells in the extracellular matrix (59). However, human neutrophil elastase is able to kill cancer cells *in vitro* and *in vivo,* which is prevented by SLPI via inactivation of neutrophil elastase (60). This suggests that protease inhibition by SLPI in the tumor microenvironment can both inhibit and promote cancer. Finally, we observed that high SLPI expression is only associated with reduced disease recurrence in stage III patients treated with adjuvant 5FU-based chemotherapy, suggesting that SLPI may predict response to adjuvant chemotherapy. SLPI may make tumors more accessible to chemotherapy by providing blood supply to hypoxic regions (39). In addition,

chemotherapy can cause a strong inflammatory response due to necrosis of cancer cells and surrounding tissue, which may promote tumor growth and could be inhibited by SLPI (54). However, whether the chemotherapy induced inflammatory response is disadvantageous or beneficial to the host is controversial (51).

4. CONCLUSIONS

SLPI protects against inflammation in the intestine. We found that high colonic epithelial SLPI expression identifies pediatric IBD patients with active clinical disease and strong immune activation characterized by extensive neutrophil infiltration and IL-17A production. Therefore, our data argue that SLPI identifies IBD patients with a strong antimicrobial immune response, which may be related to the underlying pathogenesis. Moreover, high colonic epithelial SLPI expression was associated with previously identified immune profiles related to therapy resistance in IBD, suggesting that SLPI could aid in the identification of IBD patients with therapy nonresponse. Based on the data described in this thesis and the known functions of SLPI, we anticipate that both epithelial and myeloid SLPI production in the intestine during IBD is beneficial to the host. However, experiments using mouse models for intestinal inflammation with conditional knockout for SLPI epithelial or monocytic cells are needed to test these hypotheses.

SLPI can both exert functions promoting and inhibiting cancer. We found that high SLPI expression in CRC liver metastases is associated with poor prognosis after resection of liver metastases, suggesting that detection of SLPI protein expression could aid in predicting prognosis in CRC patients with metastatic disease. In contrast, in patients with stage III MSS CRC, SLPI expression was associated with reduced recurrence of disease. These findings argue that the role of SLPI in CRC may depend on the stage of tumor progression. The association between SLPI expression in CRC liver metastases and poor prognosis and the previously identified role of SLPI in metastasis formation suggest that SLPI expression in CRC may be advantageous for metastatic tumor cells. However, the precise role of SLPI in CRC remains to be established and the results described in this thesis warrant further mechanistic analyses to investigate how SLPI promotes or counteracts human CRC. Therefore, modulation of SLPI expression in human CRC cell lines and orthotopic mouse models for CRC tumor growth and metastasis are needed.

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Appendices

I. ENGLISH SUMMARY

The immune system in the intestine

The intestine is continuously exposed to large amounts of bacteria (the commensal microbiota), which are harmless and essential for the development and function of the intestine. As a consequence, the immune system must prevent pro-inflammatory immune responses to these commensals, while mounting host defense against micro-organisms which breach the intestinal barrier. Thus, the intestinal immune response needs to be tightly regulated and tailored to the type and location of bacteria.

The intestinal epithelium, a single layer of cells between the intestinal lumen and the intestinal wall, is crucial in orchestrating this tailored immune response as it provides a physical and biochemical barrier between micro-organisms and most immune cells. In addition, the intestinal epithelium recognizes microbe-associated molecular patterns, resulting in activation of the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which leads to production of chemokines, pro-inflammatory cytokines and anti-microbial peptides important for host defense. However, to prevent continuous pro-inflammatory immune responses upon recognition of harmless bacteria, intestinal epithelial cells and other innate immune cells acquire a state of hyporesponsiveness to commensal-derived bacterial components. One important mechanism responsible for this hyporesponsiveness is negative regulation of the NF-κB pathway. In the absence of signals of danger, NF-κB inhibitors prevent intestinal inflammation by keeping innate immune cells, including intestinal epithelial cells, in a hyporesponsive state. This is essential for intestinal homeostasis, as hyporesponsive innate immune cells in turn promote a tolerogenic adaptive immune response.

The protein Secretory Leukocyte Protease Inhibitor (SLPI)

Secretory Leukocyte Protease Inhibitor (SLPI) is an evolutionary conserved protein which is expressed by many human epithelia and some innate immune cells. SLPI has many different functions and its main functions are related to the innate immune response. SLPI inhibits NF-κB and thereby regulates the acquisition and maintenance of intestinal epithelial hyporesponsiveness. In addition, SLPI inhibits proteases produced by immune cells, thereby preventing tissue damage during an inflammatory response. Moreover, SLPI has antibacterial, antifungal and antiviral properties. Thus, SLPI protects against inflammation, tissue damage and infection. Another reason to study SLPI expression in the intestine is the fact that SLPI expression is induced in epithelial cells after repetitive microbial interaction. As the intestine in particular is exposed to large

amounts of microbial antigens, we hypothesized that SLPI regulates host-microbial interactions in the intestine.

First, we questioned whether the function of SLPI is different between tissues. In **chapter 2** we review the literature on SLPI's functions in the intestine, the lungs, the skin and the vagina, as these tissues all form a barrier between the body and micro-organisms. We conclude that the varied functions of SLPI have not been systematically studied in all tissues. In the lungs, SLPI is known as an important protease inhibitor, whereas in the skin and oral mucosa SLPI has been shown to promote wound healing. In the vaginal fluid SLPI has a role in the prevention of HIV-1 transmission. We hypothesize that SLPI may exert immune regulatory functions in the lungs, skin and vagina as well.

Several studies demonstrate that SLPI is overexpressed in different types of cancer. Therefore, we questioned whether SLPI may have a function in cancer. In **chapter 2** we review the literature on SLPI's expression and function in cancer and conclude that most studies indicate that SLPI may promote in the metastatic potential of epithelial tumors. However, SLPI can both promote and prevent cancer via diverse mechanisms, depending on the type of cancer and the stage of tumor development and progression.

Inflammatory bowel disease (IBD)

Inflammatory bowel disease (IBD), comprising Crohn's disease and ulcerative colitis, is a chronic disease of the gastrointestinal tract driven by an inappropriate inflammatory T-cell response to commensal microbiota. Both Crohn's disease and ulcerative colitis patients are a heterogeneous group with varying course of disease and response to therapy. The treatment of IBD is focused on suppression of the immune system, but up to 40% of patients does not respond or loses response to treatment and suffers from relapsing disease. Immunosuppressive therapy can also lead to serious side effects. Therefore, predictors of disease course and therapy response are needed to prevent over- and undertreatment of IBD patients.

We anticipated that classification of IBD patients based on their underlying immune response will ultimately lead to better prediction of disease behavior and therapy response. The underlying immune defects in IBD patients are heterogeneous, as the innate immune response has a dual role in the pathogenesis of IBD. On the one hand, hyperresponsive innate immune cells can promote pro-inflammatory T-cell responses. On the other hand, insufficient antimicrobial host defense can result in infiltration of bacteria in the tissue, in turn leading to pro-inflammatory T cell responses to the

persisting bacteria. However, methods to identify IBD patients with insufficient anti-microbial immune responses are scarce.

As intestinal epithelial SLPI expression is induced by repetitive microbial contact, we hypothesized that high intestinal epithelial SLPI expression reflects increased contact between epithelial cells and micro-organisms and that SLPI expression is increased in the intestine of IBD patients. In **chapter 3** we demonstrate that colonic SLPI expression is increased when microbial contact is intensified. Colonic SLPI expression is increased both in the colon of mice with a genetic defect in mucus production leading to infiltration of bacteria in the tissue, and in wild-type mice with colitis due to a chemical disruption of the intestinal barrier. In addition, we show that epithelial SLPI expression is increased in the colon of IBD patients using two independent cohorts of therapy-naive pediatric IBD patients. As expression of colonic epithelial SLPI is highly variable between IBD patients, we hypothesized that high SLPI expression could identify a subgroup of patients with a distinctive underlying immune disease. We demonstrate that high colonic epithelial SLPI expression as detected with immunohistochemistry identifies pediatric IBD patients with high clinical disease activity and strong immune activation characterized by extensive neutrophil infiltration and IL-17A production. In particular, high colonic epithelial SLPI expression was associated with more severe endoscopic and microscopic disease and high numbers of infiltrating neutrophils in the tissue. Using RNA sequencing, we found that neutrophil activation and IL-17 signaling were increased in the colon of IBD patients with high colonic epithelial SLPI expression. As this subgroup of IBD patients also had a Th17 immune protein profile in the peripheral blood, our data show that colonic SLPI expression not only gives information on the colon, but also on the type of immune response in the individual patient. Thus, we demonstrate that histological detection of a single protein can be used for classification of IBD patients. In addition, gene expression in colonic biopsies from the subgroup of IBD patients with high SLPI expression was enriched for pathways associated with therapy resistance, suggesting that SLPI could aid in tailored treatment strategies in IBD.

SLPI is not only expressed by epithelial cells, but also by human macrophages, neutrophils and mast cells. In the small intestine, epithelial SLPI expression is low, but in lamina propria cells SLPI is more abundant. Therefore, we questioned which immune cells in the human intestinal lamina propria express SLPI. In **chapter 4**, we demonstrate that SLPI is expressed by monocytes or macrophages in the intestinal lamina of healthy controls and patients with IBD and that SLPI is expressed by monocytic cells. Monocytes need to adapt to the local tissue environment after migration from the bloodstream. As exogenous SLPI has been shown to be taken up by monocytes

and to regulate monocyte activation via NF-κB inhibition, we hypothesized that endogenous SLPI expression by monocytes could also regulates monocyte activation. We demonstrate that endogenous SLPI inhibits NF-κB activation in human monocytes, leading to suppression of chemokine and pro-inflammatory cytokine production. These findings imply that monocytes can self-regulate their activation upon microbial stimulation via upregulation of SLPI expression and that expression of SLPI by other cell types, such as epithelial cells, is not required for inhibition of monocyte activation. In addition, our data suggest that endogenous SLPI expression in monocytes may prevent inflammation and tissue damage during inflammation in mucosal tissues.

Colorectal cancer (CRC)

As SLPI expression is relatively high in colonic epithelial cells and is increased in several types of cancer, we questioned whether SLPI expression is increased in colorectal cancer (CRC). CRC is the fourth leading cause of cancer-related mortality. After resection of the primary tumor, patient survival is highly variable even among patients with similar clinical risk factors. Therefore, prognostic factors are needed to select patients which will benefit from additional therapy after surgery, such as chemotherapy.

The liver is the most common site of distant metastasis in CRC and resection of the affected liver can improve survival in a subgroup of CRC patients. However, also in this patient group prognostic factors are needed to identify which patients will benefit from additional therapy after resection of liver metastases.

We questioned whether SLPI expression in CRC tumor cells could predict patient prognosis and hypothesized that high SLPI expression in CRC tumor cells is related to a high metastatic potential. In **chapter 5** we assess the prognostic value of SLPI in CRC patients with liver metastases who underwent resection of liver metastases. We show that high SLPI expression in CRC liver metastases is associated with significantly shorter overall survival after resection of the liver metastases. The prognostic value of SLPI in CRC is independent of previously established clinical risk factors. In addition, we found that SLPI expression in the liver metastases is associated with SLPI expression in the matched primary tumor and that high SLPI expression in the primary tumor is also associated with significantly shorter overall survival after resection of liver metastases. These data indicate that detection of SLPI expression may help to predict the prognosis of patients with CRC after resection of liver metastases.

In **chapter 6** we assess the prognostic value of SLPI in CRC patients without distant metastases. In stage II CRC (patients without lymph node metastases), SLPI

expression is not associated with prognosis. In stage III CRC (patients with lymph node metastases), we found an association between high SLPI expression in micro-satellite stable tumors and reduced disease recurrence in patients treated with adjuvant chemotherapy, independently of previously established clinical risk factors. Together, these data suggest that SLPI may promote tumor growth in patients once distant metastases have established, but that SLPI may be unfavorable for tumors in stage III CRC. In addition, high SLPI expression in stage III CRC may predict a better response to adjuvant chemotherapy.

II. NEDERLANDSE SAMENVATTING

Het afweersysteem in de darmen

De darmen worden continu blootgesteld aan grote hoeveelheden bacteriën (het darmmicrobioom), welke onschadelijk zijn en bovendien nodig zijn voor de ontwikkeling en functie van de darmen. Daarom is het van belang dat er geen ontstekingsreactie gericht tegen deze commensalen optreedt, terwijl er wel een ontstekingsreactie moet zijn wanneer micro-organismen de darmbarrière doorbreken. Het is dus van groot belang dat de afweerreactie in de darmen goed gereguleerd wordt en afgestemd is op het type en de locatie van de aanwezige bacteriën.

De slijmvliescellen in de darm, de darmepitheelcellen, vormen één cellaag tussen het lumen van de darm (de buitenwereld) en de darmwand en zijn een fysieke en biochemische barrière tussen het darmmicrobioom en de meeste afweercellen. Daarnaast kan het darmepitheel micro-organisme-geassocieerde moleculaire patronen herkennen, waardoor een signaleringsroute kan worden geactiveerd via transcriptie factor NF-κB. Dit leidt vervolgens tot de productie van ontstekingsstoffen en signaalmoleculen die nodig zijn voor een afweerreactie. Echter, om te voorkomen dat er continu ontstekingsreacties optreden tegen onschadelijke darmbacteriën reageren darmepitheelcellen en andere afweercellen van het aangeboren afweersysteem nauwelijks op de structuren van de commensalen. Een belangrijk mechanisme wat hier verantwoordelijk voor is is de remming van de NF-κB signaleringsroute. In de afwezigheid van 'signalen van gevaar' (zoals een micro-organisme wat een infectie kan veroorzaken), zorgen NF-κB remmers er in de cel van darmepitheelcellen en andere afweercellen van het aangeboren afweersysteem voor dat er minder signaalmoleculen worden geproduceerd. Dit proces is essentieel voor een evenwichtige relatie tussen de darm en het microbioom (homeostase), omdat de remming van activatie van afweercellen van het aangeboren afweersysteem er vervolgens ook voor zorgt dat de cellen verworven afweersysteem, waaronder T cellen, de onschadelijke bacteriën tolereren.

Het eiwit Secretory Leukocyte Protease Inhibitor (SLPI)

Secretory Leukocyte Protease Inhibitor (SLPI) is een evolutionair geconserveerd eiwit wat tot expressie komt in veel verschillende typen slijmvliescellen (epitheelcellen) en sommige afweercellen van het aangeboren afweersysteem. SLPI heeft veel verschillende functies en de belangrijkste functies zijn gerelateerd aan de aangeboren afweerreactie. SLPI is een remmer van de NF-κB signaleringsroute en zorgt er daardoor voor dat darmepitheelcellen en andere cellen van het aangeboren afweersysteem nauwelijks reageren op het microbioom. Daarnaast remt SLPI eiwitsplitsende enzymen (proteasen) die door afweercellen worden gemaakt tijdens een ontstekingsreactie en

voorkomt daarmee de afbraak van weefsel. Tenslotte heeft SLPI ook antibacteriële, antischimmel en antivirale werking. SLPI beschermt dus tegen ontsteking, weefselschade en infecties. Een andere reden om de expressie van SLPI in de darmen te bestuderen is het feit dat de expressie van SLPI in epitheel wordt geïnduceerd door herhaaldelijk contact met bacteriën. Omdat de darmen worden blootgesteld aan grote hoeveelheden bacteriële antigenen, is onze hypothese dat SLPI de interactie tussen het afweersysteem en het microbioom in de darmen reguleert.

Eerst stelden we de vraag of de functie van SLPI verschillend is tussen verschillende weefsels. In **hoofdstuk 2** bediscussiëren we de literatuur over de functie van SLPI in de darmen, de longen, de huid en de vagina, omdat deze weefsels allemaal een barrière vormen tussen het lichaam en micro-organismen. We concluderen dat de gevarieerde functies van SLPI niet systematisch zijn onderzocht in elk type weefsel. In de longen is SLPI een belangrijke remmer van proteasen, terwijl SLPI in de huid en in het mondslijmvlies wondheling bevordert. In de vagina draagt SLPI bij aan het voorkomen van infectie met HIV-1. Mogelijk heeft SLPI ook afweer-regulerende functies in de longen, huid en vagina, zoals in de darmen, maar dat moet nog onderzocht worden.

Verschillende studies hebben laten zien dat SLPI verhoogd tot expressie komt in verschillende vormen van kanker. Daarom stelden we de vraag of SLPI ook een functie heeft in kanker. In **hoofdstuk 2** bespreken we de literatuur over de expressie en functie van SLPI in kanker en concluderen we dat de meeste studies laten zien dat SLPI het vormen van uitzaaiingen van epitheliale tumoren bevordert. SLPI kan echter zowel kanker bevorderen als voorkomen via verschillende mechanismen, afhankelijk van het type kanker en de fase van tumor ontwikkeling en progressie.

Chronische darmontsteking (Inflammatory Bowel Disease)

Chronische darmontsteking, in het Engels *Inflammatory Bowel Disease* (IBD), is een verzamelnaam voor de ziekte van Crohn en colitis ulcerosa. IBD is een chronische ziekte van het darmstelsel, waarbij een afwijkende reactie van inflammatoire T cellen op onschadelijke darmbacteriën tot ontsteking leidt. Ook binnen de groep patiënten met de ziekte van Crohn en binnen de groep patiënten met colitis ulcerosa verschilt de ziekte sterk tussen patiënten, aangezien het beloop van de ziekte verschillend kan zijn en de reactie op de behandeling sterk uiteen kan lopen. De behandeling van patiënten met IBD is gericht op het onderdrukken van het afweersysteem, maar tot 40% van de patiënten reageert niet of verliest de respons op behandeling en krijgt last van terugkerende ziekte en eventuele complicaties. Aan de andere kant kan afweer-onderdrukkende therapie ook ernstige bijwerkingen hebben. Daarom is het belangrijk om factoren te vinden die bij diagnose kunnen voorspellen hoe de ziekte

bij een individuele patiënt gaat verlopen en hoe de patiënt op therapie gaat reageren, zodat over- en onder-behandeling van patiënten met IBD kan worden voorkomen.

Wij veronderstelden dat classificatie van patiënten met IBD op basis van de onderliggende afweerreactie uiteindelijk zal leiden tot betere voorspelling van ziektegedrag en therapierespons. De onderliggende defecten in het afweersysteem bij patiënten met IBD zijn verschillend van patiënt tot patiënt. Zo heeft het aangeboren afweersysteem twee verschillende rollen in het ontstaan van de ziekte. Aan de ene kant kunnen cellen van het aangeboren afweersysteem die sterk reageren op darmbacteriën namelijk leiden tot inflammatoire T-cel activiteit. Aan de andere kant kan een onvoldoende afweerreactie echter ook leiden tot infiltratie van bacteriën in het weefsel, waardoor er uiteindelijk ook een inflammatoire T-cel reactie optreedt. Er zijn echter op dit moment nauwelijks methoden om IBD-patiënten met een onvoldoende aangeboren afweer op te sporen.

Aangezien expressie van SLPI in het darmepitheel geïnduceerd wordt door herhaaldelijk contact met het microbioom was onze hypothese dat hoge expressie van SLPI in het darmepitheel een reflectie is van toegenomen contact tussen epitheelcellen en micro-organismen en dat de expressie van SLPI toegenomen is in het darmepitheel van patiënten met IBD. In **hoofdstuk 3** laten we met behulp van muismodellen zien dat SLPI verhoogd tot expressie komt in de dikke darm wanneer het contact met het microbioom is toegenomen. Expressie van SLPI is verhoogd in de dikke darm van muizen met een genetisch defect in de slijmproductie in de darm, waardoor de bacteriën niet op afstand kunnen worden gehouden en de muizen een ontsteking van de dikke darm ontwikkelen. Ook vonden we verhoogde expressie van SLPI in de dikke darm van muizen waarbij de darmbarrière chemisch kapot is gemaakt en er een ontstekingsreactie optreedt tegen de infiltrerende bacteriën. Deze resultaten laten zien dat expressie van SLPI verhoogd is wanneer het contact met normaal onschadelijke darmbacteriën is toegenomen. Vervolgens laten we zien dat expressie van SLPI in het epitheel van de dikke darm verhoogd is bij patiënten met IBD vergeleken met patiënten zonder IBD, in twee onafhankelijke cohorten van therapie-naïeve kinderen met IBD. Omdat de expressie van SLPI in de dikke darm van patiënten met IBD heel variabel was tussen patiënten, was onze hypothese dat hoge expressie van SLPI een subgroep van IBD-patiënten zou kunnen identificeren met een bepaalde onderliggende afweerreactie. We laten zien dat hoge expressie van SLPI in het epitheel van de dikke darm, gedetecteerd door middel van immuunhistochemie, IBD-patiënten met hoge klinische ziekteactiviteit en sterke activatie van het afweersysteem kan identificeren. Hoge expressie van SLPI in het epitheel van de dikke darm was namelijk geassocieerd met endoscopisch en microscopisch ernstigere ziekte en een hoog aantal

neutrofiele granulocyten in het weefsel. Door te kijken naar genexpressie vonden we dat ook de activatie van neutrofiele granulocyten en de IL-17 signaleringsroute waren toegenomen in de dikke darm van IBD-patiënten met hoge expressie van SLPI in het epitheel van de dikke darm. Omdat we bij de IBD-patiënten in deze subgroep in het bloed een profiel vonden van een subtype T cellen die veel IL-17A produceren, de Th17 cellen, laten onze data zien dat expressie van SLPI in het epitheel van de dikke darm niet alleen informatie geeft over de dikke darm, maar ook over het type afweerreactie in de individuele patiënt. Concluderend laten onze bevindingen zien dat detectie van één eiwit gebruikt kan worden voor het classificeren van IBD-patiënten. Daarnaast vonden we in de patiënten met hoog SLPI in het epitheel van de dikke darm in biopten van de dikke darm een genexpressieprofiel wat geassocieerd is met therapie resistentie. Dit suggereert dat detectie van SLPI kan helpen bij het voorspellen van de reactie op therapie in patiënten met IBD.

Expressie van SLPI komt naast epitheelcellen ook voor in macrofagen, neutrofiele granulocyten en mestcellen. In de dunne darm is de expressie van SLPI in het epitheel relatief laag, maar is er wel expressie van SLPI in cellen in de lamina propria. Daarom stelden wij de vraag welke cellen van het afweersysteem SLPI tot expressie brengen in de darmen. In **hoofdstuk 4** laten we zien dat SLPI tot expressie komt in monocyten of macrofagen in de lamina propria van de darmen van gezonde controles en patiënten met IBD en dat SLPI tot expressie komt in monocytaire cellen. Monocyten moeten zich aanpassen aan de lokale omgeving van het weefsel waar ze naar toe migreren uit de bloedbaan. Omdat eerder is aangetoond dat exogeen (door andere cellen geproduceerd) SLPI de activatie van monocyten reguleert, was onze hypothese dat endogene expressie van SLPI in monocyten de activatie van monocyten kan reguleren. We laten zien dat endogeen SLPI de activatie van NF-κB remt in humane monocyten en dat dit leidt tot minder productie van signaalmoleculen en ontstekingsstoffen. Deze bevindingen impliceren dat monocyten zelf hun activatie na microbieel contact kunnen reguleren via expressie van SLPI en dat monocyten daarvoor niet afhankelijk zijn van andere SLPI-producerende cellen, zoals epitheelcellen. Daarnaast suggereren onze data dat endogene expressie van SLPI in monocyten ontsteking en weefselschade kan voorkomen tijdens afweerreacties in slijmvliezen.

Dikkedarmkanker

Aangezien expressie van SLPI relatief hoog is in het epitheel van de dikke darm en is verhoogd in verschillende typen van kanker, stelden we de vraag of expressie van SLPI verhoogd is in dikkedarmkanker. Dikkedarmkanker is de vierde grootste veroorzaker van sterfte aan kanker. Na het verwijderen van de darmtumor verschilt de overlevingsduur van patiënt tot patiënt, ook als patiënten dezelfde klinische

risicofactoren hebben. Daarom is het nodig om voorspellers te vinden van overleving, zodat de patiënten met dikkedarmkanker die baat hebben bij extra behandeling na de operatie, zoals chemotherapie, kunnen worden geselecteerd en behandeld.

De lever is de meest voorkomende plek van uitzaaiingen bij dikkedarmkanker. Bij een deel van de patiënten kan het aangedane stuk lever worden verwijderd, wat resulteert in langere overleving. Echter ook in deze patiëntengroep zijn voorspellers nodig om te weten welke patiënten baat hebben bij aanvullende behandeling na het verwijderen van de uitzaaiingen in de lever.

Wij hebben onderzocht of de expressie van SLPI in dikkedarmtumoren en in de kankercellen in uitzaaiingen in de lever de overlevingsduur van patiënten kan voorspellen. Onze hypothese was dat expressie van SLPI in dikke darmkanker ongunstig is voor het beloop van de ziekte en gerelateerd is aan meer kans op uitzaaiingen. In **hoofdstuk 5** onderzoeken we de expressie van SLPI in patiënten met dikkedarmkanker waarbij uitzaaiingen in de lever zijn verwijderd. We laten zien dat de expressie van SLPI verhoogd is in uitzaaiingen in de lever van patiënten met dikke darmkanker en dat dit geassocieerd is met een significant kortere overleving na verwijdering van de aangedane lever. De voorspellende waarde van SLPI was onafhankelijk van reeds bekende risicofactoren. Daarnaast hebben we gevonden dat expressie van SLPI in de lever uitzaaiingen overeenkomt met expressie van SLPI in de dikkedarmtumor van dezelfde patiënt en dat de expressie van SLPI in de dikkedarmtumor ook geassocieerd is met een significant kortere overleving na verwijdering van de aangedane lever. Deze data laten zien dat detectie van SLPI kan helpen bij het voorspellen van de overlevingsduur bij patiënten met dikkedarmkanker na het verwijderen van uitzaaiingen in de lever.

In **hoofdstuk 6** onderzoeken we de voorspellende waarde van expressie van SLPI bij patiënten met dikkedarmkanker zonder uitzaaiingen op afstand. Bij patiënten met stadium II dikkedarmkanker (geen uitzaaiingen) bleek expressie van SLPI niet geassocieerd te zijn met de prognose. Bij patiënten met stadium III dikkedarmkanker (uitzaaiingen in de lymfeklieren, maar niet op afstand) vonden we een associatie tussen hoge expressie van SLPI in een subgroep van de tumoren en minder terugkeer van de ziekte na operatie en chemotherapie, onafhankelijk van reeds bestaande risicofactoren. Samengenomen suggereren deze data dat SLPI een verschillende rol heeft gedurende de stadia van dikkedarmkanker. Wanneer uitzaaiingen op afstand al gevormd zijn, heeft SLPI-expressie mogelijk een voordeel voor de tumorcellen, terwijl bij patiënten met alleen lymfekliermetastasen SLPI mogelijk juist nadelig is voor de tumor. Daarnaast is het mogelijk dat expressie van SLPI bij patiënten met stadium III dikkedarmkanker een betere respons op chemotherapie na de operatie voorspelt.

III. ACKNOWLEDGEMENTS

First, I would like to thank Johannes, who helped me with all kinds of aspects of my PhD, ranging from making dots the right size in Illustrator to keeping me motivated. Without you, my figures would definitely look a lot less elegant.

Janneke, our journey together started with intestinal stromal cells in 2012 and is still ongoing with intestinal epithelial cells and neutrophils. You gave me the opportunity to start a PhD in your lab when it was the right time for me and you made sure that my PhD involved pathology. You taught me everything in the lab from how to perform experiments, how to stay positive with disappointing results and how to write scientific papers. I am also very grateful that you gave me the opportunity to go to Oxford and York, which was a valuable time for me.

I would like to thank everyone who has worked in the lab Kindergeneeskunde during my internships and my PhD. Linda, I was honored to be your paranymph and I look forward to having you next to me during my defense. I am always inspired by your perseverance to overcome hurdles and your enthusiasm. Léa, you taught me so much and it was a great pleasure to chat with you about everything from mouse breedings to worm houses. Irma, it is always great fun spending time with you and I was so lucky to share a room with you again in Delft last year! Sharon, you were my daily supervisor in 2014 and you have always stayed an example for me. Ytje, you lent me the SLPI project and we started to understand each other better every year. I especially enjoyed learning R together with you and talking to you about life outside the lab. You do not take things for granted and you never do things halfway! Dicky, working with you was always a great pleasure and I feel lucky that I learned immunohistochemistry from you. Daniëlle van Haaften, I enjoyed working with you very much, together we were able to stain many slides at once and I appreciate how you are always interested in everyone's stories. Rolien, thank you for helping with every project. It was great to share the love for film festivals, concerts and the combination black and purple. Lisette, thank you for teaching me how to deal with mice! It was always nice to discuss De Mol with you. Celia, thank you for your work on the SLPI project and for answering all our questions about DSS. Marieke, thank you for supervising me during my first internship in the lab. Lilian, it has been a long time but I still remember your friendly help. Beatriz, thank you for working with so much enthusiasm on the RNA sequencing data. Bas, thank you for your help in the lab; your humor and jokes bring more fun to the group. Wilco, thank you for helping me with finding lost datapoints. Maud and Daniëlle Barendregt, thank you for working together on PIBD-SETQ; it was great when you joined our room. Mo, I was lucky to have your friendly help with the analyses. Brenda, it was a pleasure to

meet you and I hope you found a great next adventure. Nina, I enjoyed supervising you and I was impressed how you stayed motivated during the pandemic.

Marcel, you do so much for the lab but most of all I enjoyed your stories at the coffee table. Theo, your stories were also very enjoyable and it was a pleasure to crochet Mycoplasma pneumoniae for you. Lisa, I am glad we still find time every now and then to have a cup of tea together. Ruben, you inspired me with your motivation and of course the toasted baguette with cheese. Wendy, Silvia, Ad, Ana and Jop: thank you for your help and the nice atmosphere in the lab.

Hankje, Lissy, Barbara, Myrthe, Martine, Renz, Lotte, Merel, Martha and Willie: thank you for the interesting meetings and valuable collaboration. It was refreshing to get out of the lab every now and then and hear about the actual patients.

Tom, thank you for the useful meetings, the Thursday 9:00 was my favorite meeting of the week. I am also grateful for connecting me to the STROMA project. Natalie, Mónica, Julien, Ferry, Madelon and Zoltan: it was great to share meetings, antibodies and dinners with you!

Gerrit, Remond, Pien, Jeroen, Sjoerd, Joyce, Hein, Jan and Menno: thank you for the pleasant collaboration on SLPI in colorectal cancer. I enjoyed every trip to the NKI.

Dimitris, thank you for answering all my questions about statistics, even when I kept asking for more clarification. Mathijs, Gregory and Eric, thank you for collaborating on the RNA sequencing data.

I would like to thank all pathologist in the Erasmus MC, PAL Dordrecht and the Reinier de Graaf Gasthuis Delft who supported me when finishing my thesis during my training. In particular I would like to thank Folkert, Arno and Rob for giving me the opportunity to interrupt my training for so many years. In addition, I would like to thank Katharina and Michail for their input in the IBD project.

I am also grateful to all pathology trainees with whom I shared activities in the last years. Eva, our first year together was unforgettable and although you were much faster than I am, I am happy that you will also be my paranymph! Charlotte, we will always remember how paper can mimic a tumor. Your humor makes daily things more fun!

Kevin, thank you for being my supervisor in Oxford, it was fantastic to be part of your group. I very much appreciated the time you took for scientific discussions and social

activities. Mark, thank you for letting me work on SLPI in York, you gave me a lot of freedom in the lab.

Ruud, thank you for your coaching. Your questions helped me stay on track when I was finishing my thesis.

I would like to thank Frank van Vliet and the Molmed postgraduate school for organizing the research master and many great courses.

To all my friends: thank you for your endless support! A special thanks to Malou, Serdar, Hessel and Rachel Paterson for sharing your experiences.

Cedric, you wrote about the curvy road that led to your thesis but also about being satisfied with the end result, which encouraged me to take this route. Simone, I was impressed by your adventurous PhD project and I really like your thesis cover. Henk, you made sure I would not forget the propositions. It was special to attend your defense and I think I may have copied your attention for detail. Marian, I am happy that your painting is on the cover of this thesis and that you always stimulated me to do what I like most.

IV. PHD PORTFOLIO

Course / congress	Organizer	ECTS
Molmed Day Erasmus MC (2017)	Erasmus MC, MolMed	0.30
NVGE conference (2017)	Nederlandse Vereniging voor Gastro-Enterologie (NVGE)	0.60
Animal course (article 9) (2017)	Erasmus MC Animal Facility	4.50
ESPGHAN master class (2017)	European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN)	0.60
NVVI annual meeting (2017), **oral presentation**	Nederlandse Vereniging voor Immunologie (NVVI)	0.60
Postgraduate Course Advanced Immunology (2018)	Amsterdam UMC	3.00
Molmed Day Erasmus MC (2018), **oral presentation**	Erasmus MC, MolMed	0.30
NVVI symposium (2018)	Nederlandse Vereniging voor Immunologie (NVVI)	0.60
Medical Immunology Course (2018)	Erasmus MC	1.50
Academic Network Meeting & Maastricht Pathology meeting (2018)	The Pathological Society of Great Britain and Ireland & the British Division of the International Academy of Pathology (BDIAP)	1.50
Mucosal Immunology Course & Symposium (2018), **poster presentation & travel award**	Society for Mucosal Immunology	1.20
European Conference on Immunology Amsterdam (2018), **oral presentation & travel award**	European Federation of Immunological Societies	1.20
Basic course in R (2018)	Erasmus MC, MolMed	1.80
Scientific Integrity (2018)	Erasmus MC Graduate School	0.30
Junior Academy Deutsche Gesellschaft für Pathologie (2018), **oral presentation**	Deutsche Gesellschaft für Pathologie	1.20
Pathologendagen najaar (2018), **oral presentation on pathology training**	Nederlandse Vereniging voor Pathologie (NVVP)	0.60
Molmed Day Erasmus MC (2019), **poster presentation**	Erasmus MC, MolMed	0.30
NVVI symposium (2019)	Nederlandse Vereniging voor Immunologie (NVVI)	0.60
Pathologendagen voorjaar (2019)	Nederlandse Vereniging voor Pathologie (NVVP)	0.90
Academic Network Meeting & Leeds Pathology meeting (2019), **thesis pitch (2nd price), poster presentation & travel grant British Division of the International Academy of Pathology (BDIAP)**	The Pathological Society of Great Britain and Ireland & the BDIAP	1.20

PhD portfolio (Continued)

Course / congress	Organizer	ECTS
Gene expression data analysis using R: How to make sense out of your RNA-Seq/microarray data (2019)	Erasmus MC, MolMed	2.00
Pathologendag najaar (2019)	Nederlandse Vereniging voor Pathologie (NVVP)	0.30
NVVI annual meeting (2019), **poster presentation**	Nederlandse Vereniging voor Immunologie (NVVI)	0.60

Other activities	Organizer	ECTS
Teaching at the Erasmus University College: 3x guest lecture on the histology and function of the gastrointestinal tract (2018, 2019 and 2020)	Erasmus University College (EUC)	0.60
President of the Dutch society for pathology trainees (2018-2020)	Landelijke Pathologie Assistenten Vereniging (LPAV)	3.00
Board member of the Dutch society for pathology (2018 - 2020)	Nederlandse Vereniging voor Pathologie (NVVP)	6.00
Supervision of an Infection & Immunity research master student (February 2020 - December 2020)	Laboratory of Pediatrics	6.00
Total ECTS		------ + **41.30**

V. LIST OF PUBLICATIONS

Included in this thesis

Endogenous secretory leukocyte protease inhibitor inhibits microbial-induced monocyte activation
Sandrine Nugteren, Ytje Simons-Oosterhuis, Celia L Menckeberg, Danielle H Hulleman-van Haaften, Dicky J Lindenbergh-Kortleve, Janneke N Samsom
European Journal of Immunology, 2023 Feb;53(2):e2249964. doi: 10.1002/eji.202249964.

High expression of Secretory Leukocyte Protease Inhibitor (SLPI) in stage III microsatellite stable colorectal cancer is associated with reduced disease recurrence
Sandrine Nugteren, Sjoerd H. den Uil, Pien M. Delis-van Diemen, Ytje Simons-Oosterhuis, Dicky J. Lindenbergh-Kortleve, Daniëlle H. van Haaften, Hein B.A.C. Stockmann, Joyce Sanders, Gerrit A. Meijer, Remond J.A. Fijneman, Janneke N. Samsom
Scientific Reports, 2022 Jul 16;12(1):12174. doi: 10.1038/s41598-022-16427-5.

Secretory Leukocyte Protease Inhibitor (SLPI) in mucosal tissues: Protects against inflammation, but promotes cancer
Sandrine Nugteren, Janneke N. Samsom
Cytokine and Growth Factor Reviews, 2021 Jun;59:22-35. doi: 10.1016/j.cytogfr.2021.01.005.

Expression of the immune modulator secretory leukocyte protease inhibitor (SLPI) in colorectal cancer liver metastases and matched primary tumors is associated with a poorer prognosis
Sandrine Nugteren, Jeroen A.C.M. Goos, Pien M. Delis-van Diemen, Ytje Simons-Oosterhuis, Dicky J. Lindenbergh-Kortleve, Daniëlle H. van Haaften, Joyce Sanders, Gerrit A. Meijer, Remond J.A. Fijneman & Janneke N. Samsom
Oncoimmunology, 2020 Oct 13;9(1):1832761. doi: 10.1080/2162402X.2020.1832761.

Other publications

Duplication of the IL2RA locus causes excessive IL-2 signaling and may predispose to very early onset colitis
Maria E. Joosse, Fabienne Charbit-Henrion, Remy Boisgard, Rolien (H.) C. Raatgeep, Dicky J. Lindenbergh-Kortleve, Léa M. M. Costes, **Sandrine Nugteren**, Nicolas Guegan, Marianna Parlato, Sharon Veenbergen, Valérie Malan, Jan K. Nowak, Iris H. I. M. Hollink, M. Luisa Mearin, Johanna C. Escher, Nadine Cerf-Bensussan and Janneke N. Samsom
Mucosal Immunology, 2021 Sep;14(5):1172-1182. doi: 10.1038/s41385-021-00423-5.

CRP-point of care test (POCT): when is it necessary?
S. Nugteren, H.J.C.M. Pleumeekers, J.A.H. Eekhof
Huisarts en Wetenschap, 2015;58(6):322-6.

Nudging in the Dutch society?
S. Nugteren, M. Jansen, S. Tirband Dastgerdi, L. Persoon, H. Tursucu, J. Polder, F. van der Lucht
Tijdschrift voor Gezondheidswetenschappen, 2012; 90: 20–22. doi: 10.1007/s12508-012-0014-z.

Why do HIV-1 vaccines not work?
S. Nugteren, S. Samiei, A. de Goede, R. Gruters
Erasmus Journal of Medicine, 2010 May.

VI. ABOUT THE AUTHOR

Sandrine Nugteren was born in 1990 in Dordrecht, The Netherlands. She lived in Lusaka, Zambia, until she was almost four years old and moved to Dordrecht. Sandrine became interested in biomedical research when she joined the first edition of the Junior Med School at the Erasmus Medical Center (EMC) in Rotterdam. In 2008 she moved to Rotterdam to study Medicine at the EMC and in 2010 she started a research master in Infection & Immunity at the EMC. Sandrine was co-founder of the Student Union for Research Masters (SURE). As part of the research master, Sandrine visited Oxford, the UK, for an internship and stayed in the UK for a Marie Curie project at the University of York. During her medical internships Sandrine missed studying diseases at the cell level, which led to elective internships in the pathology department and in December 2015 the start of her training in the pathology department at the EMC. After one year she interrupted her clinical training for four years to perform a PhD in the Laboratory of Pediatrics at the EMC. Currently, Sandrine is continuing her training in pathology and is planning an additional training in clinical molecular biology in pathology. Sandrine lives in the city center of Rotterdam next to one of the hidden city gardens.