Check for updates



15214141, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/eji.202249964 by Erasmus University Rotterdam Universiteitsbibliotheek, Wiley Online Library on [20/01/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

European Journal of Immunology Clinical·Basic·Translational

Molecular immunology and signaling

Basic

Research Article

# 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 and Janneke N. Samsom***

Laboratory of Pediatrics, division Gastroenterology and Nutrition, Erasmus University Medical Center, Rotterdam, The Netherlands

**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 NF 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 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.**

**Keywords:** SLPI · monocytes · NF-κB · immune regulation · intestine

Additional supporting information may be found online in the Supporting Information section at the end of the article.

## 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

*Correspondence:* Dr. Janneke N. Samsom
e-mail: j.samsom@erasmusmc.nl

© 2022 The Authors. *European Journal of Immunology* published by Wiley-VCH GmbH



This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-No Derivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

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 the 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 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 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 humans 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.

## 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 [27, 28] and from the database of immune cell expression, expression quantitative trait loci, and epigenomics (DICE) project [29]. 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 h 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 an inhibitor of NF-κB, we measured mRNA expression of the other known NF-κB inhibitors TNF-α-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) (Fig. 1c-h). A double staining for SLPI and CD68 indicated that these SLPI-positive cells are monocytes or macrophages (Fig. 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.

© 2022 The Authors. *European Journal of Immunology* published by Wiley-VCH GmbH



15214141, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/eji.202249964 by Erasmus University Rotterdam Universiteitsbibliotheek, Wiley Online Library on [20/01/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

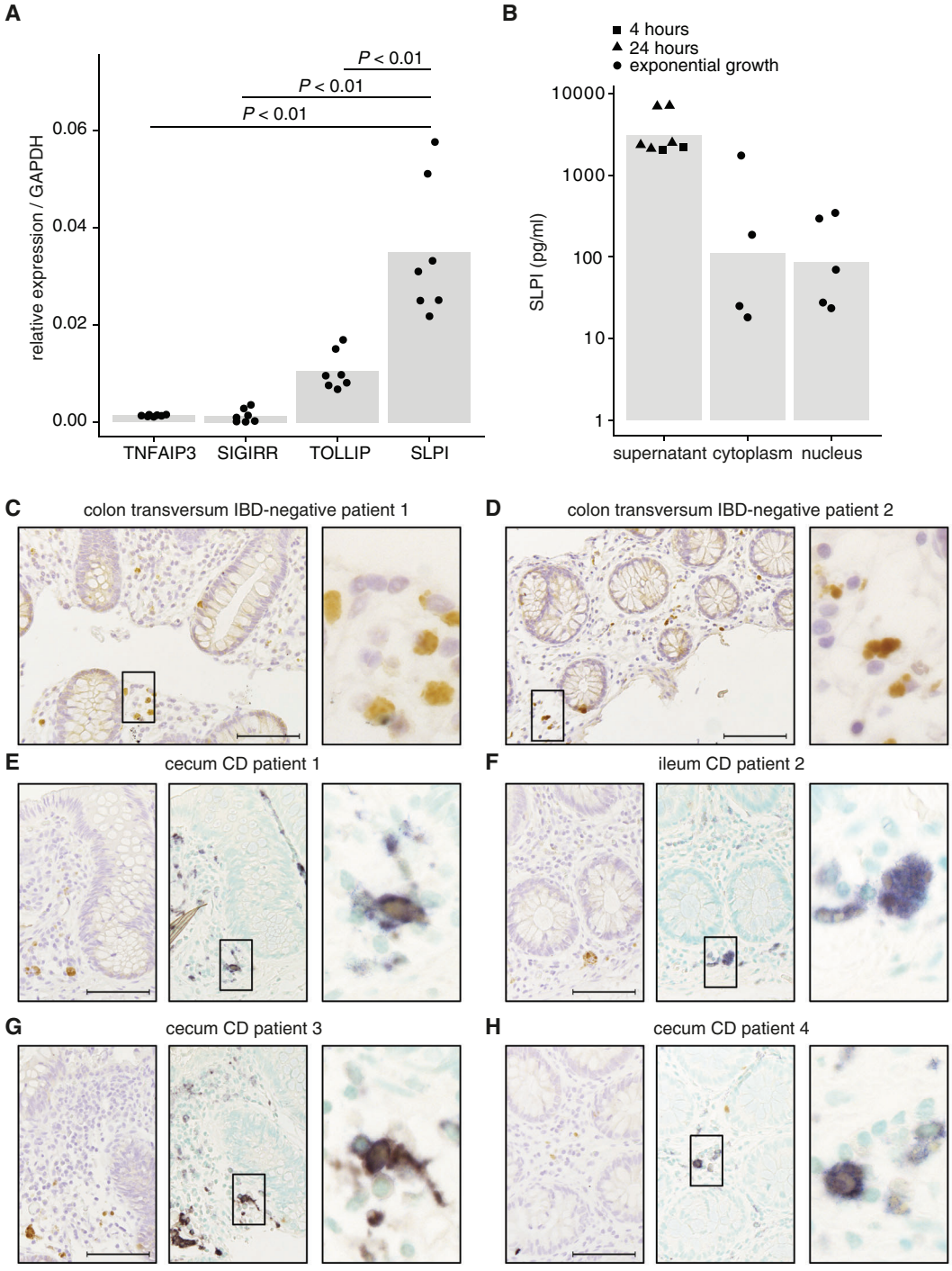


**Figure 1. SLPI is produced by THP-1 cells and is expressed by mononuclear cells in the human intestinal lamina propria.** (a) mRNA expression of NF-κB inhibitors *TNFAIP3*, *SIGIRR*, *TOLLIP*, and *SLPI* was measured by qPCR in wild-type THP-1 cells after 24 h of culture without stimulation. P-values 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 wild-type THP-1 cells harvested in their exponential growth phase (black dots), and in supernatant of wild-type 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 of 15 pediatric patients without inflammatory bowel disease (IBD-negative) (c and d) and in resection material of 10 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 Figure S1a and b.

© 2022 The Authors. *European Journal of Immunology* published by Wiley-VCH GmbH



15214141, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/eji.202249964 by Erasmus University Rotterdam Universiteitsbibliotheek, Wiley Online Library on [20/01/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

## 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 and 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 wild-type THP-1 cells (Figure 2c and d; Figure S2a and b). In addition, pre-incubation with IFN-γ, which upregulates TLR expression, and subsequent LPS-stimulation increased CXCL8 and TNF-α production up to sixfold in SLPI-deficient THP-1 cells compared to wild-type control (Figure 2c and d; Figure S2a and 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 wild-type THP-1 cells, LPS stimulation increased phosphorylated NF-κB in the nucleus and phosphorylated IκBα levels in the cytoplasm after 15 min (Figure 2e and 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 and f). These data show that endogenous SLPI inhibits NF-κB signaling in monocytic cells in response to microbial signals.

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 that lacks the signal peptide and is thought to stay intracellularly [18, 30]. 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; Figure S3a and 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 wild-type levels, confirming that SLPI directly suppresses CXCL8 production in THP-1 cells (Figure 3b; Figure S3c). Similarly, reconstitution with truncated SLPI also decreased LPS-induced CXCL8 production to wild-type 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 min after LPS stimulation (Figure 3c and 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 min after LPS stimulation, we could not detect reduced IκBα phosphorylation after reconstitution with truncated SLPI in multiple experiments (Figure 3c and 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 an 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.

## 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, 31]. However, we found that endogenous SLPI is also able to suppress 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 wild-type THP-1 cells (Figures 1b and 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 wild-type 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 wild-type THP-1 cells and SLPI-deficient THP-1 cells (Figure 4b and c). However, LPS stimulation in the presence

© 2022 The Authors. *European Journal of Immunology* published by Wiley-VCH GmbH



15214141, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/eji.202249964 by Erasmus University Rotterdam Universiteitsbibliotheek, Wiley Online Library on [20/01/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

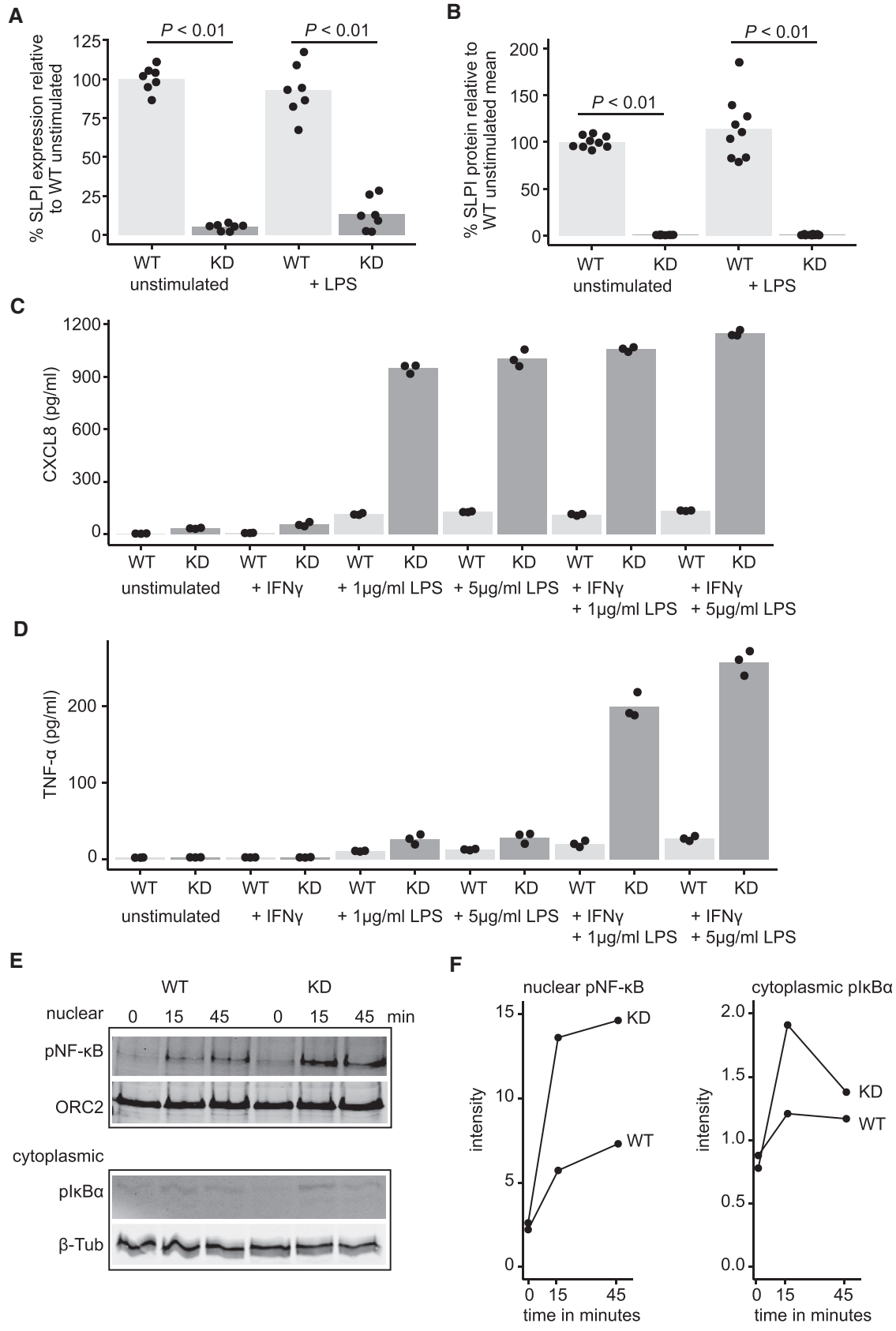


**Figure 2. Knockdown of SLPI in THP-1 cells increases CXCL8 and TNF-α production via NF-κB signaling in response to microbial stimulation.** THP-1 cells transduced with virus supernatant containing an empty LZRS-IRES-EGFP vector (wild-type, WT) or an empty LZRS-IRES-EGFP vector

© 2022 The Authors. *European Journal of Immunology* published by Wiley-VCH GmbH



15214141, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/eji.202249964 by Erasmus University Rotterdam Universiteitsbibliotheek, Wiley Online Library on [20/01/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

of exogenous recombinant SLPI did not inhibit CXCL8 release in wild-type or SLPI knockdown THP-1 cells at either of the time-points, suggesting that exogenous SLPI is unable to inhibit THP-1 cell activation despite reaching the nucleus and the cytoplasm (Figure 4b and 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.

## 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 the 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 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 [32]. 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 [33]. In addition, PLSCR1 modulates phagocytosis and can be detected in fully internalized phagosomes [33, 34]. 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 [27, 28]). 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

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 h 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 h 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 h of culture for measurement of CXCL8 protein production by ELISA. (d) Supernatant was collected after 6 h 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 Figure S1a and b). 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 (wild-type, 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 min (e + f). Images of the blots (e) and densitometry of the protein bands relative to loading controls (f) are shown.

© 2022 The Authors. *European Journal of Immunology* published by Wiley-VCH GmbH



15214141, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/eji.202249964 by Erasmus University Rotterdam Universiteitsbibliotheek, Wiley Online Library on [20/01/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

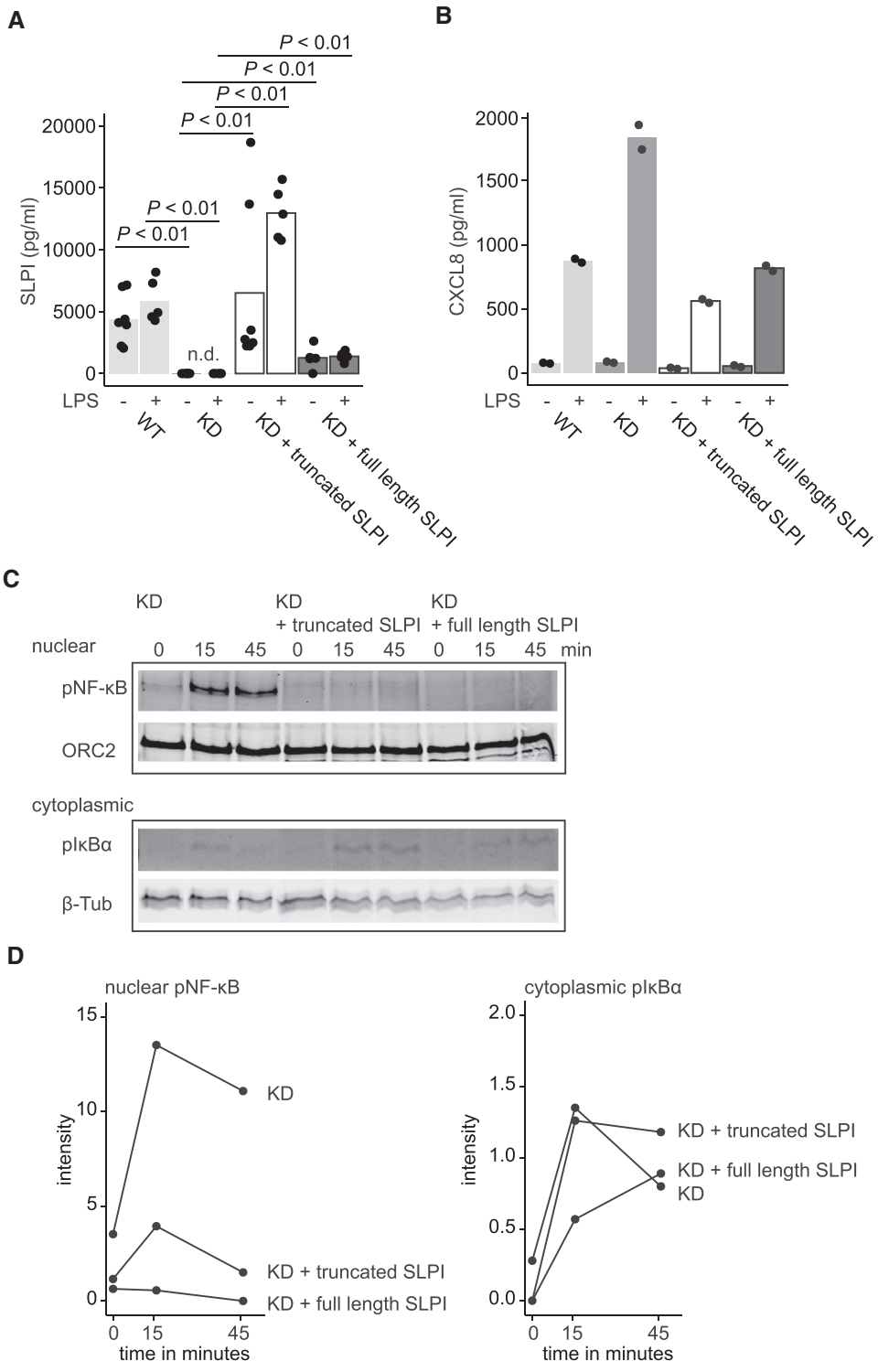


**Figure 3. Reconstitution of truncated or full length SLPI in THP-1 cells rescues chemokine inhibition in response to microbial stimulation.** THP-1 cells were transduced with virus supernatant containing either an empty LZRS-IRES-EGFP vector (wild-type, WT), or shRNA specific for SLPI in a pRSC vector 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 Figure S3c). 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 min (c + d). Images of the blots (c) and densitometry of the protein bands relative to loading controls are shown (d). n.d. = not detectable.

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.

© 2022 The Authors. *European Journal of Immunology* published by Wiley-VCH GmbH



15214141, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/eji.202249964 by Erasmus University Rotterdam Universiteitsbibliotheek, Wiley Online Library on [20/01/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

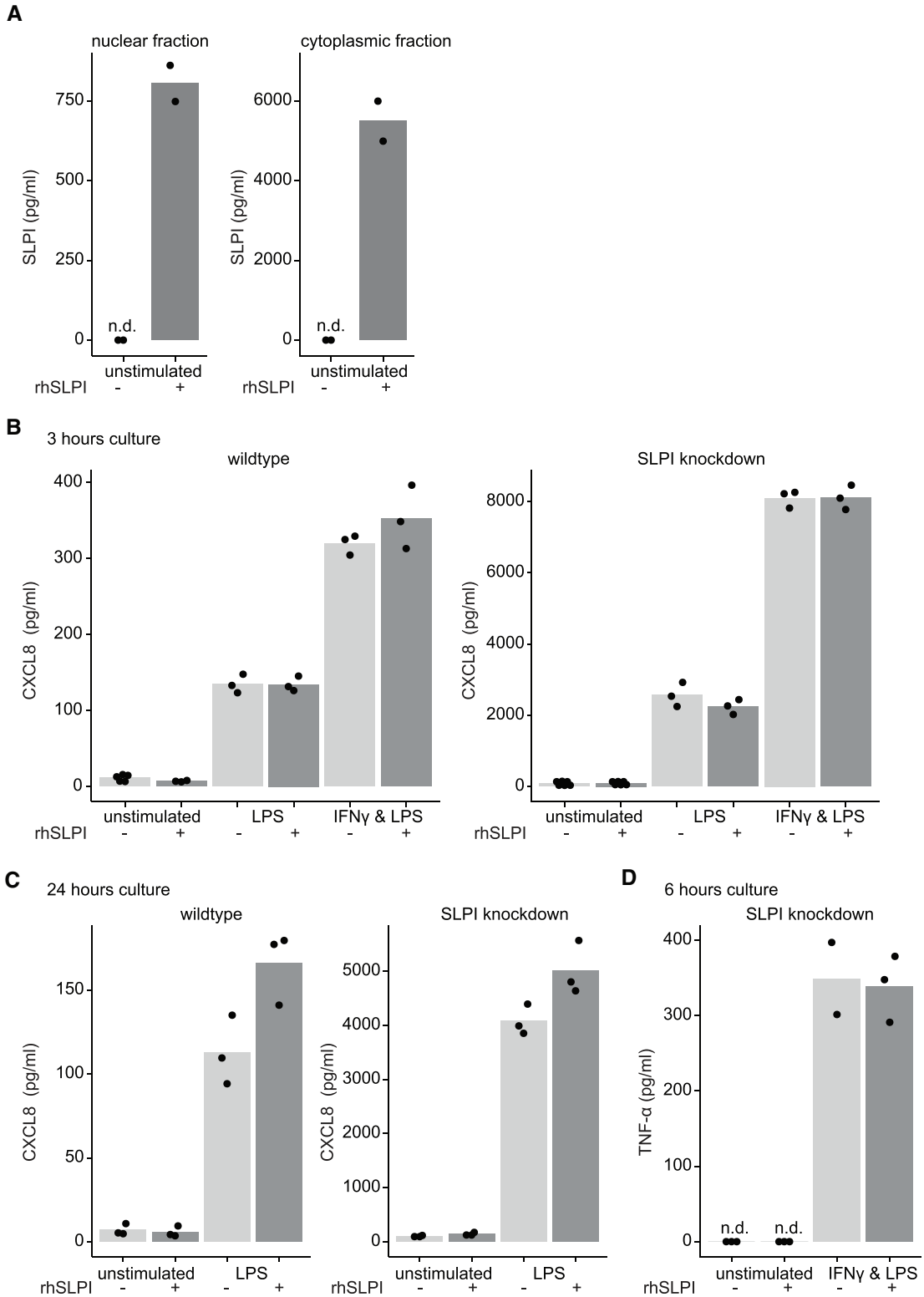


**Figure 4. Exogenous SLPI does not regulate LPS-induced CXCL8 production by THP-1 cells**. Recombinant human SLPI (10 μg/mL) was added to the culture of THP-1 cells transduced with virus supernatant containing shRNA specific for SLPI in a pRSC vector (SLPI knockdown). (a) SLPI protein was measured by 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). Wild-type 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 h before stimulation with LPS (b + c + d). Supernatant

© 2022 The Authors. *European Journal of Immunology* published by Wiley-VCH GmbH



15214141, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/eji.202249964 by Erasmus University Rotterdam Universiteitsbibliotheek, Wiley Online Library on [20/01/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

## 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 [35]. 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 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 BLOX-ALL (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 (Figure S1a) and one section was incubated with PBS (Figure S1b: 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 20× brightfield lens (Leica 506503) and a 63× 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 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

was collected after 3 hours of culture (b) or 24 h of culture (c) for measurement of CXCL8 protein by ELISA. Supernatant was collected after 6 h of culture for measurement of TNF-α protein by ELISA (d). TNF-α protein was not detectable in the supernatant of wild-type 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.

© 2022 The Authors. *European Journal of Immunology* published by Wiley-VCH GmbH



15214141, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/eji.202249964 by Erasmus University Rotterdam Universiteitsbibliotheek, Wiley Online Library on [20/01/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Cancer Institute, Amsterdam, The Netherlands) for stable RNA interference as described previously [16]. The pRSC vector is a modification of the pRETRO-SUPER [36]. 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, 30]. The DNA encoding full-length SLPI or truncated SLPI was subcloned into an 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:

ATGAAGTCCAGCGGCCTCTTCCCCTTCCTGGTGCTGCTTGCC
CTGGGAACTCTGGCACCTTGGGCTGTGGAAGGCTCTGGAAAGTC
CTTCAAAGCTGGAGTCTGTCCTCCTAAGAAATCTGCCCAGTGCCT
TAGATACAAGAAACCTGAGTGCCAGAGTGACTGGCAGTGTCCAGG
GAAGAAGAGATGTTGTCCTGACACTTGTGGCATCAAATGCCTGGA
TCCTGTTGACACCCCAAACCCAACAAGGAGGAAGCCTGGGAAGT
GCCCAGTGACTTATGGCCAATGTTTGATGCTTAACCCCCCCAATTT
CTGTGAGATGGATGGCCAGTGCAAGCGTGACTTGAAGTGTTGCA
TGGGCATGTGTGGGAAATCCTGCGTTTCCCCTGTGAAAGCTTGAT
TCCTGCCATATGGAGGAGGCTCTGGAGTCCTGCTCTGTGTGGTCC
AGGTCCTTTCCACCCTGAGACTTGGCTCCACCACTGATATCCTCC
TTTGGGGAAAGGCTTGGCACACAGCAGGCTTTCAAGAAGTGCCA
GTTGATCAATGAATAAATAAACGAGCCTATTTCTCTTTGCA

truncated SLPI:

TCTGGAAAGTCCTTCAAAGCTGGAGTCTGTCCTCCTAAGAAAT
CTGCCCAGTGCCTTAGATACAAGAAACCTGAGTGCCAGAGTGACT
GGCAGTGTCCAGGGAAGAAGAGATGTTGTCCTGACACTTGTGGC
ATCAAATGCCTGGATCCTGTTGACACCCCAAACCCAACAAGGAGG
AAGCCTGGGAAGTGCCCAGTGACTTATGGCCAATGTTTGATGCTT
AACCCCCCCAATTTCTGTGAGATGGATGGCCAGTGCAAGCGTGAC
TTGAAGTGTTGCATGGGCATGTGTGGGAAATCCTGCGTTTCCCCT
GTGAAAGCTTGATTCCTGCCATATGGAGGAGGCTCTGGAGTCCTG
CTCTGTGTGGTCCAGGTCCTTTCCACCCTGAGACTTGGCTCCACC
ACTGATATCCTCCTTTGGGGAAAGGCTTGGCACACAGCAGGCTTT
CAAGAAGTGCCAGTTGATCAATGAATAAATAAACGAGCCTATTTCT
CTTTGCA

## 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 h 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 min at 1000 × *g* at room temperature and cultured at 37°C for 2 h. This infection cycle was repeated three 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.

© 2022 The Authors. *European Journal of Immunology* published by Wiley-VCH GmbH



15214141, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/eji.202249964 by Erasmus University Rotterdam Universiteitsbibliotheek, Wiley Online Library on [20/01/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

| Human gene | Forward primer | Reverse primer |
|---|---|---|
| GAPDH | GTCGGAGTCAACGGATT | AAGCTTCCCGTTCTCAG |
| SLPI | TCCAGGGAAGAAGAGA TGT | TGCCCATGCAACACTT |
| TNFAIP3 | TGGCACAACTCATCTC ATC | 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 min. 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 min at 37°C and subsequently deactivated with 0.05 M EDTA. SLPI protein was measured by ELISA as described below. Total protein concentrations were measured using the Bradford method [37].

## 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 h at room temperature. Subsequently, samples were incubated for 2 h 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 h at room temperature and subsequently incubated with 1:5000 HRP-conjugated streptavidin (BD Biosciences; 554066) in the dark at room temperature for approximately 1 h. 3, 3′,5,5′-Tetramethylbenzidine Liquid Substrate (Sigma–Aldrich; T4444) was used for detection of the HRP. The reaction was stopped using 1 M $H_3PO_4$. 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. TBS with 0.1% TWEEN 20 (Sigma–Aldrich) and 5% non-fat dry milk or 5% BSA for the 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) |

© 2022 The Authors. European Journal of Immunology published by Wiley-VCH GmbH



15214141, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/eji.202249964 by Erasmus University Rotterdam Universiteitsbibliotheek, Wiley Online Library on [20/01/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

### 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 [38]. The package ggplot2 [39] was employed for visualization.

**Acknowledgements:** The authors 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.

**Conflict of interest:** The authors declare no commercial or financial conflict of interest.

**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) (35). This study does not include experiments using animals.

**Author contributions:** S.N. performed investigation, formal analysis, visualization, and wrote original draft. Y.S. was associated with methodology, investigation, and formal analysis. C.L.M. performed investigation, formal analysis, and reviewed and edited the final manuscript. D.H.H. and D.J.L. were associated with methodology and investigation. J.N.S. was associated with conceptualization, funding acquisition, resources, supervision, and reviewed and edited the final manuscript. All authors read and approved the final manuscript.

**Data availability statement:** The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Peer review:** The peer review history for this article is available at https://publons.com/publon/10.1002/eji.202249964

## References

1 **Maruyama, M., Hay, J. G., Yoshimura, K., Chu, C. S., Crystal, R. G.,** Modulation of secretory leukoprotease inhibitor gene expression in human bronchial epithelial cells by phorbol ester. *J Clin Invest* 1994. **94**(1): 368-375.

2 **Sallenave, J. M., Shulmann, J., Crossley, J., Jordana, M., Gauldie, J.,** Regulation of secretory leukocyte proteinase inhibitor (SLPI) and elastase-specific inhibitor (ESI/elafin) in human airway epithelial cells by cytokines and neutrophilic enzymes. *Am. J. Respir. Cell Mol. Biol.* 1994. **11**(6): 733-741.

3 **Bohm, B., Aigner, T., Kinne, R., Burkhardt, H.,** The serine-protease inhibitor of cartilage matrix is not a chondrocytic gene product. *Eur. J. Biochem.* 1992. **207**(2): 773-779.

4 **Sallenave, J. M., Si Tahar, M., Cox, G., Chignard, M., Gauldie, J.,** Secretory leukocyte proteinase inhibitor is a major leukocyte elastase inhibitor in human neutrophils. *J. Leukoc. Biol.* 1997. **61**(6): 695-702.

5 **Mihaila, A., Tremblay, G. M.,** Human alveolar macrophages express elafin and secretory leukocyte protease inhibitor. *Z. Naturforsch. C* 2001. **56**(3-4): 291-297.

6 **Westin, U., Polling, A., Ljungkrantz, I., Ohlsson, K.,** Identification of SLPI (secretory leukocyte protease inhibitor) in human mast cells using immunohistochemistry and in situ hybridisation. *Biol. Chem.* 1999. **380**(4): 489-493.

7 **Palm, E., Khalaf, H., Bengtsson, T.,** Suppression of inflammatory responses of human gingival fibroblasts by gingipains from Porphyromonas gingivalis. *Mol. Oral Microbiol.* 2015. **30**(1): 74-85.

8 **Taggart, C. C., Greene, C. M., McElvaney, N. G., O'Neill, S.,** Secretory leucoprotease inhibitor prevents lipopolysaccharide-induced IkappaBalpha degradation without affecting phosphorylation or ubiquitination. *J. Biol. Chem.* 2002. **277**(37): 33648-33653.

9 **Taggart, C. C., Cryan, S. A., Weldon, S., Gibbons, A., Greene, C. M., Kelly, E., Low, T. B., O'neill, S. J., McElvaney, N. G.,** Secretory leucoprotease inhibitor binds to NF-kappaB binding sites in monocytes and inhibits p65 binding. *J. Exp. Med.* 2005. **202**(12): 1659-1668.

10 **Thompson, R. C., Ohlsson, K.,** Isolation, properties, and complete amino acid sequence of human secretory leukocyte protease inhibitor, a potent inhibitor of leukocyte elastase. *Proc. Natl. Acad. Sci. U S A* 1986. **83**(18): 6692-6696.

11 **Hiemstra, P. S., Maassen, R. J., Stolk, J., Heinzel-Wieland, R., Steffens, G. J., Dijkman, J. H.,** Antibacterial activity of antileukoprotease. *Infect. Immun.* 1996. **64**(11): 4520-4524.

12 **Tomee, J. F., Hiemstra, P. S., Heinzel-Wieland, R., Kauffman, H. F.,** Antileukoprotease: an endogenous protein in the innate mucosal defense against fungi. *J. Infect. Dis.* 1997. **176**(3): 740-747.

13 **McNeely, T. B., Dealy, M., Dripps, D. J., Orenstein, J. M., Eisenberg, S. P., Wahl, S. M.,** Secretory leukocyte protease inhibitor: a human saliva protein exhibiting anti-human immunodeficiency virus 1 activity in vitro. *J. Clin. Invest.* 1995. **96**(1): 456-464.

14 **Vroling, A. B., Konijn, T., Samsom, J. N., Kraal, G.,** The production of secretory leukocyte protease inhibitor by dendritic cells. *Mol. Immunol.* 2011. **48**(4): 630-636.

15 **Samsom, J. N., van der Marel, A. P., van Berkel, L. A., van Helvoort, J. M., Simons-Oosterhuis, Y., Jansen, W., Greuter, M., Nelissen, R. L., Meeuwisse, C. M., Nieuwenhuis, E. E., Mebius, R. E., Kraal, G.,** Secretory leukoprotease inhibitor in mucosal lymph node dendritic cells regulates the threshold for mucosal tolerance. *J. Immunol.* 2007. **179**(10): 6588-6595.

16 **Menckeberg, C. L., Hol, J., Simons-Oosterhuis, Y., Raatgeep, H. R., de Ruiter, L. F., Lindenbergh-Kortleve, D. J., Korteland-van Male, A. M., El Aidy, S., van Lierop, P. P., Kleerebezem, M., Groeneweg, M., Kraal, G., Elink-Schuurman, B. E., de Jongste, J. C., Nieuwenhuis, E. E., Samsom, J. N.,** Human buccal epithelium acquires microbial hyporesponsiveness at birth, a role for secretory leukocyte protease inhibitor. *Gut.* 2014.

17 **Nakamura, A., Mori, Y., Hagiwara, K., Suzuki, T., Sakakibara, T., Kikuchi, T., Igarashi, T., Ebina, M., Abe, T., Miyazaki, J., Takai, T., Nukiwa, T.,** Increased susceptibility to LPS-induced endotoxin shock in secretory

© 2022 The Authors. *European Journal of Immunology* published by Wiley-VCH GmbH



15214141, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/eji.202249964 by Erasmus University Rotterdam Universiteitsbibliotheek, Wiley Online Library on [20/01/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

leukoprotease inhibitor (SLPI)-deficient mice. *J. Exp. Med.* 2003. **197**(5): 669-674.

18 **Jin, F. Y., Nathan, C., Radzioch, D., Ding, A.**, Secretory leukocyte protease inhibitor: a macrophage product induced by and antagonistic to bacterial lipopolysaccharide. *Cell* 1997. **88**(3): 417-426.

19 **Ding, A., Thieblemont, N., Zhu, J., Jin, F., Zhang, J., Wright, S.**, Secretory leukocyte protease inhibitor interferes with uptake of lipopolysaccharide by macrophages. *Infect. Immun.* 1999. **67**(9): 4485-4489.

20 **McNeely, T. B., Shugars, D. C., Rosendahl, M., Tucker, C., Eisenberg, S. P., Wahl, S. M.**, Inhibition of human immunodeficiency virus type 1 infectivity by secretory leukocyte protease inhibitor occurs prior to viral reverse transcription. *Blood* 1997. **90**(3): 1141-1149.

21 **Greene, C. M., McElvaney, N. G., O'Neill, S. J., Taggart, C. C.**, Secretory leucoprotease inhibitor impairs Toll-like receptor 2- and 4-mediated responses in monocytic cells. *Infect. Immun.* 2004. **72**(6): 3684-3687.

22 **Desalegn, G., Pabst, O.**, Inflammation triggers immediate rather than progressive changes in monocyte differentiation in the small intestine. *Nat. Commun.* 2019. **10**(1): 3229-019-11148-2.

23 **Smythies, L. E., Sellers, M., Clements, R. H., Mosteller-Barnum, M., Meng, G., Benjamin, W. H., Orenstein, J. M., Smith, P. D.**, Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity. *J. Clin. Invest.* 2005. **115**(1): 66-75.

24 **Smith, P. D., Smythies, L. E., Shen, R., Greenwell-Wild, T., Gliozzi, M., Wahl, S. M.**, Intestinal macrophages and response to microbial encroachment. *Mucosal. Immunol.* 2011. **4**(1): 31-42.

25 **Bergenfeldt, M., Nystrom, M., Bohe, M., Lindstrom, C., Polling, A., Ohlsson, K.**, Localization of immunoreactive secretory leukocyte protease inhibitor (SLPI) in intestinal mucosa. *J. Gastroenterol.* 1996. **31**(1): 18-23.

26 **Si-Tahar, M., Merlin, D., Sitaraman, S., Madara, J. L.**, Constitutive and regulated secretion of secretory leukocyte proteinase inhibitor by human intestinal epithelial cells. *Gastroenterology* 2000. **118**(6): 1061-1071.

27 **Uhlén, M., Fagerberg, L., Hallström, B. M., Lindskog, C., Oksvold, P., Mardinoglu, A., Sivertsson, Å., Kampf, C., Sjöstedt, E., Asplund, A., Olsson, I., Edlund, K., Lundberg, E., Navani, S., Szigyarto, C. A., Odeberg, J., Djureinovic, D., Takanen, J. O., Hober, S., Alm, T., Edqvist, P. H., Berling, H., Tegel, H., Mulder, J., Rockberg, J., Nilsson, P., Schwenk, J. M., Hamsten, M., von Feilitzen, K., Forsberg, M., Persson, L., Johansson, F., Zwahlen, M., von Heijne, G., Nielsen, J., Pontén, F.**, Proteomics. Tissue-based map of the human proteome. *Science* 2015. **347**(6220): 1260419.

28 **Uhlen, M., Karlsson, M. J., Zhong, W., Tebani, A., Pou, C., Mikes, J., Lakshmikanth, T., Forsström, B., Edfors, F., Odeberg, J., Mardinoglu, A., Zhang, C., von Feilitzen, K., Mulder, J., Sjöstedt, E., Hober, A., Oksvold, P., Zwahlen, M., Ponten, F., Lindskog, C., Sivertsson, Å., Fagerberg, L., Brodin, P.**, A genome-wide transcriptomic analysis of protein-coding genes in human blood cells. *Science* 2019. **366**(6472): eaax9198.

29 **Schmiedel, B. J., Singh, D., Madrigal, A., Valdovino-Gonzalez, A. G., White, B. M., Zapardiel-Gonzalo, J., Ha, B., Altay, G., Greenbaum, J. A., McVicker, G., Seumois, G., Rao, A., Kronenberg, M., Peters, B., Vijayanand, P.**, Impact of Genetic Polymorphisms on Human Immune Cell Gene Expression. *Cell* 2018. **175**(6): 1701-1715.e16.

30 **Zitnik, R. J., Zhang, J., Kashem, M. A., Kohno, T., Lyons, D. E., Wright, C. D., Rosen, E., Goldberg, I., Hayday, A. C.**, The cloning and characterization of a murine secretory leukocyte protease inhibitor cDNA. *Biochem. Biophys. Res. Commun.* 1997. **232**(3): 687-697.

31 **Guerrieri, D., Tateosian, N. L., Maffia, P. C., Reiteri, R. M., Amiano, N. O., Costa, M. J., Villalonga, X., Sanchez, M. L., Estein, S. M., Garcia, V. E., Sallenave, J. M., Chuluyan, H. E.**, Serine leucocyte proteinase inhibitor-treated monocyte inhibits human CD4(+) lymphocyte proliferation. *Immunology* 2011. **133**(4): 434-441.

32 **Tsuchiya, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T., Tada, K.**, Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). *Int. J. Cancer* 1980. **26**(2): 171-176.

33 **Py, B., Basmaciogullari, S., Bouchet, J., Zarka, M., Moura, I. C., Benhamou, M., Monteiro, R. C., Hocini, H., Madrid, R., Benichou, S.**, The phospholipid scramblases 1 and 4 are cellular receptors for the secretory leukocyte protease inhibitor and interact with CD4 at the plasma membrane. *PLoS One* 2009. **4**(3): e5006.

34 **Herate, C., Ramdani, G., Grant, N. J., Marion, S., Gasman, S., Niedergang, F., Benichou, S., Bouchet, J.**, Phospholipid Scramblase 1 Modulates FcR-Mediated Phagocytosis in Differentiated Macrophages. *PLoS One* 2016. **11**(1): e0145617.

35 **Aardoom, M. A., Kemos, P., Tindemans, I., Aloi, M., Koletzko, S., Levine, A., Turner, D., Veereman, G., Neyt, M., Russell, R. K., Walters, T. D., Ruemmele, F. M., Samsom, J. N., Croft, N. M., de Ridder, L.**, PIBD-SETQuality consortium and PIBD-NET. International prospective observational study investigating the disease course and heterogeneity of paediatric-onset inflammatory bowel disease: the protocol of the PIBD-SETQuality inception cohort study. *BMJ Open* 2020. **10**(7): e035538-2019-035538.

36 **Brummelkamp, T. R., Bernards, R., Agami, R.**, A system for stable expression of short interfering RNAs in mammalian cells. *Science* 2002. **296**(5567): 550-553.

37 **Bradford, M. M.**, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 1976. **72**: 248-254.

38 R Core Team, *R Foundation for Statistical Computing*, Vienna, Austria. R: A Language and Environment for Statistical Computing. 2018; Available at: https://www.R-project.org, version 3.5.1.

39 **Wickham, H.**, ggplot2: Elegant Graphics for Data Analysis. 2016; Available at: https://ggplot2.tidyverse.org.

*Abbreviations:* **CXCL8**: C-X-C motif chemokine ligand 8 · **IκBα**: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha · **NF-κB**: nuclear factor kappa light chain enhancer of activated B cells · **ORC2**: Origin Recognition Complex subunit 2 · **SLPI**: Secretory Leukocyte Protease Inhibitor

*Full correspondence:* Dr. Janneke N. Samsom, PhD, Erasmus University Medical Center-Sophia Children's Hospital, Laboratory of Pediatrics, division Gastroenterology and Nutrition, Room Ee1567A, P.O. Box 2040, 3000 CA Rotterdam, the Netherlands. e-mail: j.samsom@erasmusmc.nl

Received: 24/4/2022
Revised: 6/11/2022
Accepted: 7/12/2022
Accepted article online: 8/12/2022

© 2022 The Authors. *European Journal of Immunology* published by Wiley-VCH GmbH



15214141, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/eji.202249964 by Erasmus University Rotterdam Universiteitsbibliotheek, Wiley Online Library on [20/01/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License