

ORIGINAL ARTICLE

Human buccal epithelium acquires microbial hyporesponsiveness at birth, a role for secretory leukocyte protease inhibitor

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ABSTRACT

Objective Repetitive interaction with microbial stimuli renders epithelial cells (ECs) hyporesponsive to microbial stimulation. Previously, we have reported that buccal ECs from a subset of paediatric patients with Crohn's disease are not hyporesponsive and spontaneously released chemokines. We now aimed to identify kinetics and mechanisms of acquisition of hyporesponsiveness to microbial stimulation using primary human buccal epithelium.

Design Buccal ECs collected directly after birth and in later stages of life were investigated. Chemokine release and regulatory signalling pathways were studied using primary buccal ECs and the buccal EC line TR146. Findings were extended to the intestinal mucosa using murine model systems.

Results Directly after birth, primary human buccal ECs spontaneously produced the chemokine CXCL-8 and were responsive to microbial stimuli. Within the first weeks of life, these ECs attained hyporesponsiveness, associated with inactivation of the NF- κ B pathway and upregulation of the novel NF- κ B inhibitor SLPI but no other known NF- κ B inhibitors. SLPI protein was abundant in the cytoplasm and the nucleus of hyporesponsive buccal ECs. Knock-down of SLPI in TR146-buccal ECs induced loss of hyporesponsiveness with increased NF- κ B activation and subsequent chemokine release. This regulatory mechanism extended to the intestine, as colonisation of germfree mice elicited SLPI expression in small intestine and colon. Moreover, SLPI-deficient mice had increased chemokine expression in small intestinal and colonic ECs.

Conclusions We identify SLPI as a new player in acquisition of microbial hyporesponsiveness by buccal and intestinal epithelium in the first weeks after microbial colonisation.

INTRODUCTION

Mucosal epithelial cells (ECs) are equipped with pattern recognition receptors (PRR), such as toll-like receptors (TLR) and nucleotide-binding oligomerisation domain-containing (NOD) receptors¹ that incite cellular activation after ligation of specific bacterial structures.² In healthy individuals, the interaction with commensal intestinal microbiota

Significance of this study

What is already known on this subject?

- In healthy individuals, the mucosal epithelium is hyporesponsive (tolerant) towards the commensal microflora.
- In patients with Crohn's disease and ulcerative colitis, aberrant epithelial responses to commensal microbiota contribute to chronic inflammation of the gastrointestinal tract.
- Buccal epithelium from a subset of paediatric patients with Crohn's disease, spontaneously released increased amounts of CXCL-8, CXCL-9 and CXCL-10 when compared to epithelium from healthy controls and children with ulcerative colitis.

What are the new findings?

- Neonatal buccal epithelial cells (ECs) are immunologically active and respond to microbial stimuli.
- Within the first weeks of life, buccal EC acquire hyporesponsiveness.
- SLPI drives buccal EC hyporesponsiveness through regulation of NF- κ B signalling.
- SLPI also regulates EC hyporesponsiveness to microbiota in the intestine.

How might it impact on clinical practice in the foreseeable future?

- Future research will identify whether activity of buccal epithelium may serve as a biomarker to identify a particular subset of patients with Crohn's disease who have specific alterations in innate immune regulation.

does not lead to an ensuing inflammatory response. However, upon encounter of pathogenic bacteria, PRRs convey the proinflammatory immune responses that are required to eradicate these pathogens.³ The mechanisms that account for this tailored hyporesponsiveness of ECs are a topic of extensive research.⁴ Using murine models it has been shown that ECs of neonatal mice become

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hypo-responsive to TLR stimulation immediately after birth.^{5 6} The acquisition of this hypo-responsiveness was dependent on a transient cellular activation induced by contact with exogenous TLR ligands.⁶ Transient epithelial stimulation was found to induce a negative regulatory mechanism through depleting IL-1 receptor associated kinase-1 (IRAK-1) which rendered the cells unresponsive to subsequent stimulation.^{5 6}

Acquisition of microbial hypo-responsiveness is seen in several innate cell types and can be regulated via multiple mechanisms. Functional experiments with epithelial and monocytic cells have shown that PRR signalling can be controlled by reducing the surface expression of the receptors or inhibition of mRNA synthesis.⁷ Additionally, a network of intracellular negative regulators can limit PRR-mediated overactivation. Among these, IL-1 receptor-associated kinase M (IRAK-M), NOD2, Tollip, A20, Single Immunoglobulin Interleukin-1 Receptor-related protein (SIGIRR) and SLPI have been found to inhibit intracellular signal transduction at various stages during the signalling cascade; ultimately leading to reduced NF- κ B activation and inflammatory gene expression.^{8–13}

Currently, it is unclear how human primary ECs acquire microbial hypo-responsiveness. In patients with Crohn's disease and ulcerative colitis aberrant epithelial responses to commensal microbiota contribute to chronic inflammation of the gastrointestinal tract.¹⁴ Recently, we have observed that buccal ECs from paediatric patients with Crohn's disease spontaneously released increased amounts of CXCL-8, CXCL-9, and CXCL-10 when compared to epithelium from healthy controls and children with ulcerative colitis.¹⁵ Stimulation with bacterial products resulted in a further increase of chemokine production by buccal ECs from paediatric patients with Crohn's disease, whereas the epithelium of healthy controls and patients with ulcerative colitis remained hypo-responsive to TLR stimulation. These data reveal that primary buccal ECs are a valuable tool to study acquisition of hypo-responsiveness in human primary epithelium, and may help identify dysregulation of this mechanism in Crohn's disease.

The aim of this study was to address how primary buccal ECs from healthy individuals have become hypo-responsive to TLR stimulation as a result of microbial colonisation of the intestinal tract. It is likely that the transition from the sterile in utero environment to continuous contact with microbial products at birth is a pivotal event for shaping epithelial responses. Therefore, we investigated the chemokine release and microbial responsiveness of human buccal ECs directly after birth and in later stages of life, and studied EC responses during colonisation of germ-free mice.

METHODS

Buccal EC collection

Buccal ECs were collected from at-term neonates and controls (ages indicated in figure legends). For neonates, the cells were obtained within 10 min after birth. The buccal ECs were collected by gently rubbing the inside of the cheeks with a Cytobrush Plus (CooperSurgical, Trumbull, Connecticut, USA). Buccal EC suspensions were washed twice in phosphate buffered saline (PBS) and centrifuged for 5 min at 485 g. Buccal ECs contaminated with blood detectable by microscopy were excluded from further analyses. Written informed consent was obtained from each donor or, when applicable, from the parents. The local ethics committee has approved the study protocol (also see online supplementary methods).

Cell culture and stimulation assays

LPS and PG stimulation of buccal ECs

Buccal ECs (3.5×10^4) from neonates or controls were seeded in 200 μ l medium per well of a 96-wells flat bottom plate (Corning B.V. Life Sciences, Amsterdam, The Netherlands). Buccal ECs were left untreated or were stimulated with either lipopolysaccharide (LPS) (1 μ g/mL) or peptidoglycan (PG) (10 μ g/mL) for 24 h, and supernatants were collected for enzyme linked immunosorbent assay (ELISA). The remaining buccal ECs were used for cytospin analysis or intracellular protein analysis. Cellular viability before and after culture was determined by trypan blue staining, neutral red assay and [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] (MTS) assay. To obtain cytopins, cells were resuspended in 50 μ l PBS and 50 μ l 10% human serum albumin, and spun onto microscope slides at 50 g. The slides were air dried on silica gel, and either used directly for immunohistochemical staining or stored at -20°C .

Desensitisation of TR146 cells

TR146 cells (kindly provided by Professor Mark Herzberg, University of Minnesota, USA), used at passages ranging from 3 to 15, were seeded at a density of 0.5×10^5 cells/mL in a 24-well plate (Corning B.V. Life Sciences) and cultured for 24 h, after which the cells were stimulated with PG (50 μ g/mL) for 24 h or left untreated. Supernatant was collected for ELISA. For restimulation, the cells were washed and restimulated with PG (50 μ g/mL) or medium and incubated for another 24 h, after which the supernatant was collected for ELISA. For measurements of NF- κ B activation, TR146 cells (0.5×10^6 cells/mL) were stimulated with PG (50 μ g/mL) for 24 h or left untreated. Supernatant was collected for ELISA and cells were collected for analysis of intracellular protein. The remainder of the cells was restimulated with PG for 5, 15 or 45 min, and nuclear and cytosolar cell fractions were analysed.

Statistical analysis

Statistical analysis was performed using unpaired or paired t test, t test with Welch correction, Mann-Whitney analysis, and two-way ANOVA, as indicated in the figure legends. Calculated p values are indicated in the figures.

RESULTS

Neonatal buccal ECs spontaneously produce CXCL-8 and are highly responsive to microbial stimuli

To investigate whether primary ECs from neonates are responsive to microbial stimuli, we collected buccal ECs from 14 newborns within 10 min after birth and compared their activation status with ECs from 15 controls aged 2–6 months. Neonatal buccal ECs spontaneously released substantial amounts of CXCL-8 when compared to ECs from controls (figure 1A). After 24 h of culture, buccal ECs were viable and actively responded to bacterial stimulation, as demonstrated by the additional increase in chemokine release upon incubation with LPS or PG (figure 1A; see online supplementary figure 1). Responsiveness to bacterial stimulation was restricted to neonatal buccal ECs, since neither control infant buccal ECs (2–6 month) nor buccal ECs from adults (age 29–55 years) were responsive to bacterial stimulation (figure 1A; see online supplementary figure 1). Adult buccal ECs were viable and not anergic, as shown in online supplementary figure 1.

At birth, a transition occurs from the sterile in utero environment in amniotic fluid to an environment rich in microbial

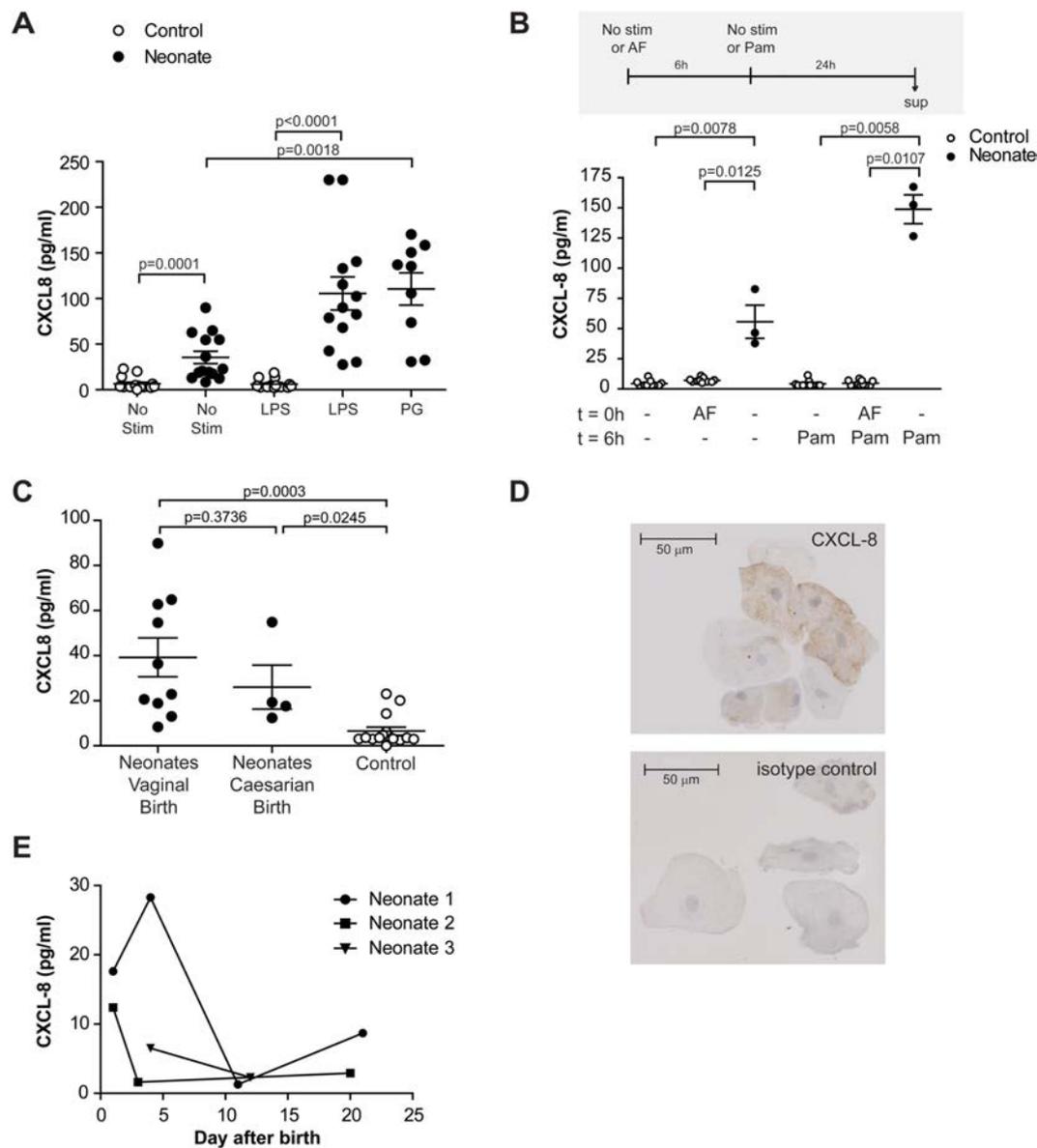


Figure 1 Neonatal buccal epithelial cells (ECs) spontaneously produce CXCL-8 and are highly responsive to microbial stimuli. (A) Buccal ECs from 14 neonates and 15 controls aged 2–6 months were collected and cultured for 24 h with or without LPS (1 μ g/mL) or PG (10 μ g/mL), and CXCL-8 release was measured by ELISA. Data are expressed as means with SEM. p Values calculated using a Mann–Whitney analysis. (B) Buccal ECs from 11 controls (age 29–55 years) were collected and incubated for 6 h with or without (–) amniotic fluid (AF). After washing, cells were cultured with or without Pam3Cys (Pam, 10 μ g/mL) for 24 h and CXCL-8 release was determined. As a control, buccal ECs from 3 neonates were incubated for 24 h with or without Pam3Cys. Data are expressed as means with SEM. p Values calculated using a Mann–Whitney analysis. (C) Buccal ECs were collected from neonates born via vaginal birth (n=10) or born by caesarian section (n=4). Buccal ECs from 15 subjects (age 2–6 months) served as control. Cells were cultured for 24 h with or without stimulus and CXCL-8 was determined in the supernatant. Cellular viability assessed by trypan blue staining and neutral red assay was not affected by 24 h in culture. Data are expressed as means with SEM. p Values calculated using a Mann–Whitney analysis. (D) CXCL-8 stain (brown, top) or isotype control (bottom) on cytopins of neonatal buccal ECs. Staining is representative for 3 different donors. (E) Buccal ECs from 3 neonates delivered by caesarian section were collected at several time points after birth. Buccal ECs of neonate 1 were collected on days 4, 11 and 21. Buccal ECs for neonate 2 were collected on days 3 and 20, and buccal ECs for neonate 3 were collected on days 4 and 12. Cells were stimulated with LPS (1 μ g/mL) for 24 h. CXCL-8 production was measured in supernatant.

products. As such, chemokine release by the epithelium directly after birth may be a remnant of activation by components in the amniotic fluid which were encountered in utero. Indeed, prenatal CXCL-8 production by intestinal ECs has been observed.¹⁶ Alternatively, the epithelium is inactive at birth and only starts releasing chemokines after microbial encounter during birth. Therefore, we assessed if 6 h preincubation with amniotic fluid alone could induce CXCL-8 production by buccal ECs from adult subjects (age 29–55 years) after 24 h of culture. Amniotic fluid incubation did not evoke CXCL-8

production (figure 1B), nor did preincubation prime sensitivity to a subsequent stimulation with Pam3Cys (figure 1B). These data strengthen the hypothesis that first microbial contact at birth initiates a temporary activation of the epithelium.

Vaginal birth is associated with ECs coming into contact with a higher number and a larger variety of microbial stimuli than birth by caesarian section. Therefore, the effect of the method of delivery on epithelial responsiveness was assessed. Spontaneous CXCL-8 release by buccal ECs from neonates delivered through vaginal birth was equal to or higher than that

from neonates delivered by caesarean section (figure 1C). Immunohistochemical staining confirmed that CXCL-8 was produced by ECs (figure 1D). To establish the kinetics with which primary buccal epithelium from neonates loses microbial responsiveness, buccal ECs from three neonates delivered by caesarean section were collected at several time points after birth and stimulated with LPS. CXCL-8 production was reduced over time in all three neonates (figure 1E). Overall, these data show that primary buccal ECs obtained directly after birth secrete chemokines, and are responsive to bacterial stimulation, whereas infant and adult buccal ECs have lost this capacity.

Priming of naive TR146 cells with peptidoglycan results in hyporesponsiveness that is associated with the expression of SLPI

As primary epithelium is short-lived, we wished to identify a putative mechanism for acquisition of epithelial hyporesponsiveness using the buccal epithelial cell line TR146. Cell lines that are initially responsive to microbial stimuli have been shown to progressively become hyporesponsive to prolonged LPS stimulation.¹⁷ Indeed, TR146 cells incubated for 24 h with PG released substantial amounts of CXCL-8 (figure 2A). This release was largely inhibited when the cells were pretreated with the same concentration of PG for 24 h (figure 2A). To identify whether hyporesponsiveness was accompanied by reduced NF- κ B activity, phosphorylated NF- κ B levels were measured in nuclear fractions as well as total and phosphorylated I κ B levels in cytosolic fractions. TR146 cells stimulated once with PG showed a clear increase in nuclear phosphorylated NF- κ B levels compared to hyporesponsive TR146 cells, indicating a more activated phenotype of the former cells (figure 2B). In contrast to cells which first encountered PG, hyporesponsive TR146 cells preincubated with PG for 24 h and subsequently restimulated with PG for 0, 5, 15 and 45 min showed no increase in nuclear translocation of NF- κ B, no reduction in cytosolic I κ B levels, and contained lower levels of phosphorylated I κ B (figure 2B).

To assess the factors involved in downregulation of the NF- κ B pathway, we first searched for known negative regulatory molecules in hyporesponsive TR146 cells. Recently, we established that SLPI is a mucosal immune regulator for microbial sensitivity by dendritic cells.¹⁸ Therefore, we cultured TR146 cells with or without stimulus and analysed changes in SLPI expression and protein production. Indeed TR146 cells expressed SLPI mRNA, spontaneously released SLPI protein in the supernatant and, upon stimulation with PG, increased their SLPI release (figure 2C, D). SLPI can act as a potent inhibitor of TLR stimulation in three ways. First, SLPI can directly interact with LPS, thus inhibiting TLR-mediated activation in responder cells.¹⁹ Second, intracellular SLPI can inhibit toll-like receptors 2 and 4 signalling by direct prevention of the degradation of the inhibitory factor I κ B α .²⁰ Third, SLPI can act in the nucleus by directly binding to an NF- κ B p65 consensus sequence in the promoter region of the CXCL-8 and TNF- α genes, thus blocking transcription.¹¹ To assess whether SLPI could also perform its intracellular inhibitory functions, we determined the SLPI concentration in cytosol and nuclear fractions and found that SLPI protein could be detected in the cytosol and the nucleus of TR146 cells (see online supplementary figure 2).

To determine the relative contribution of the inhibitory role of SLPI in hyporesponsive TR146 cells to that of other inhibitors, we compared SLPI mRNA expression to that of other known inhibitors involved in downregulation of the NF- κ B pathway. Very low levels of A20, Tollip and SIGIRR mRNA could be found in the lysates of TR146 cells by PCR analysis while

SLPI mRNA was abundantly present and could be enhanced by microbial stimulation (figure 2E).

The acquisition of hyporesponsiveness and subsequent SLPI expression were not restricted to buccal ECs only, as cells from the colonic EC line, Caco-2, responded similarly (see online supplementary figure 3). These data indicate that hyporesponsive ECs exhibit higher levels of I κ B and lower levels of phosphorylated NF- κ B and phosphorylated I κ B which correlated with increased production of SLPI protein.

Knockdown of SLPI in the buccal epithelial cell line, TR146, results in spontaneous high CXCL-8 production

To elucidate the role for SLPI in acquisition of hyporesponsiveness and loss of CXCL-8 production, endogenous SLPI was knocked down using stable shRNA expression in TR146 cells. Mock transduced cells which received the pRSC retroviral vector only, and cells which were transduced with a non-silencing sequence were used as controls. Knockdown of SLPI in the TR146 cells was successful, as demonstrated by reduced SLPI mRNA levels and SLPI secretion (figure 3A, B). Crucially, knockdown of SLPI led to spontaneously enhanced production of CXCL-8 in the knockdown cells at mRNA and protein levels when compared with mock transduced cells (figure 3A, B). SLPI knockdown also spontaneously increased CXCL-9 mRNA and CXCL-10 protein production in SLPI knockdown cells (see online supplementary figure 2). These data demonstrate that inhibition of SLPI abrogates cellular hyporesponsiveness and leads to increased chemokine production.

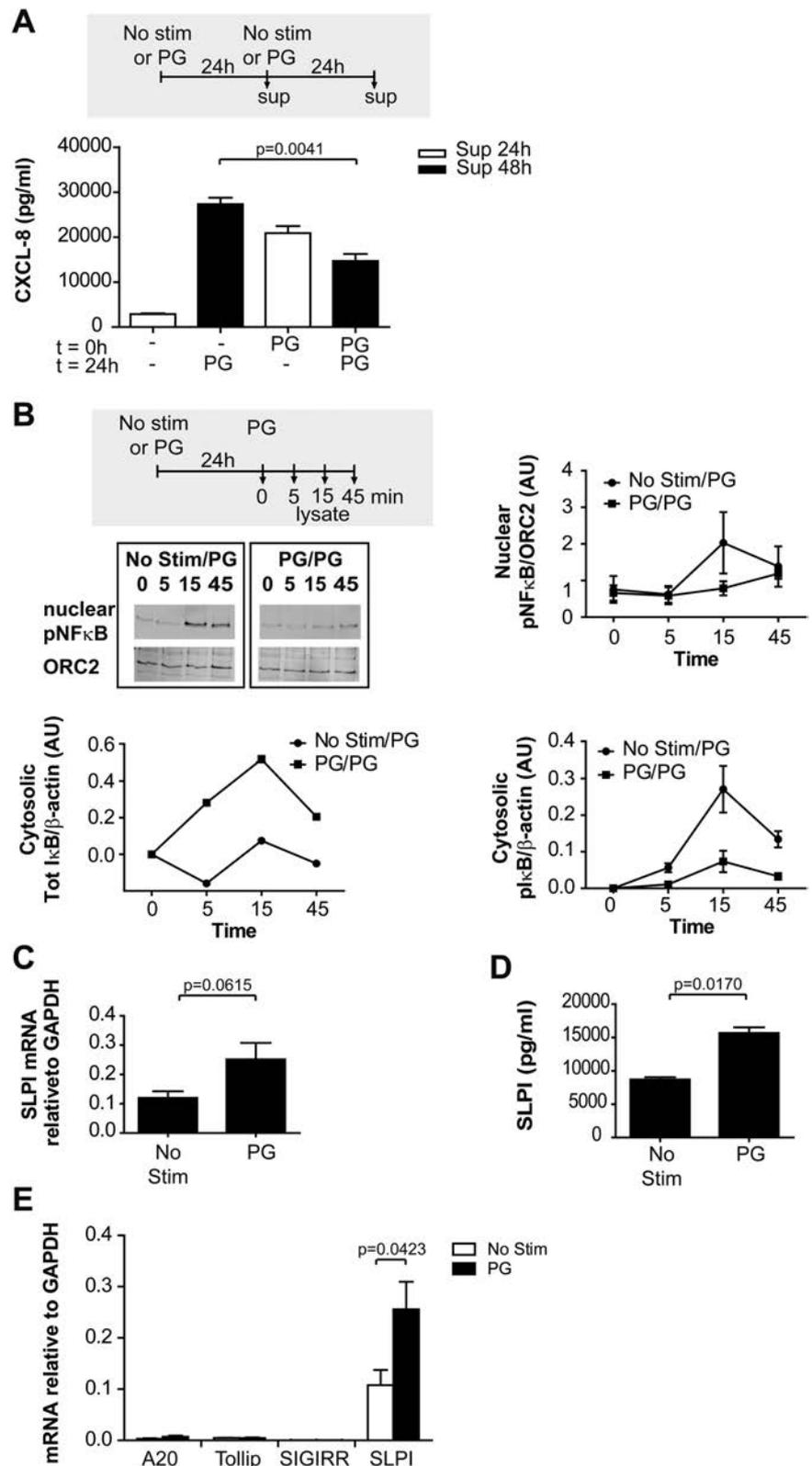
Neonatal buccal epithelial cell sensitivity to microbial stimulation is associated with decreased SLPI expression

We then examined whether hyporesponsive adult buccal ECs had a phenotypic profile that was similar to hyporesponsive TR146 cells. In agreement with the TR146 data, lysates of responsive neonatal buccal ECs had lower levels of I κ B α than adult hyporesponsive buccal ECs (figure 4A). Upon analysis of six adult donors, intracellular SLPI was consistently detectable in whole cell lysates (figure 4B). Based on the TR146 experiments described above, we hypothesised that SLPI is involved in acquiring hyporesponsiveness, and therefore wished to determine whether neonatal cells expressed low levels of SLPI. In the buccal cavity, SLPI is present as a secretory product of the salivary gland.²¹ In theory, buccal ECs could take up SLPI from their environment. Therefore, we determined the presence of SLPI mRNA in neonatal and adult buccal ECs. Hyporesponsive adult buccal ECs expressed significantly higher levels of SLPI mRNA compared to responsive neonatal buccal ECs (figure 4C). In comparison, mRNA levels for A20, Tollip and SIGIRR were very low or undetectable in neonatal and adult buccal ECs (figure 4D).

As SLPI has been shown to exert its immunosuppressive activities at different cellular sites, we further analysed the cellular location of SLPI protein in buccal ECs from adults. Crucially, SLPI was found abundantly in the nuclear fraction (figure 4E, F) of adult buccal ECs. As expected, SLPI was also located within the cytosolic fraction of adult ECs (figure 4F). On multiple occasions, cytosolic SLPI was not detected by western blot analysis (figure 4E), since the detection limit of this assay was approximately 25 ng as assessed with recombinant SLPI.

In summary, hyporesponsive buccal ECs from adult individuals exhibit high levels of I κ B compared with responsive neonatal buccal ECs. Moreover, adult buccal ECs produce high levels of SLPI mRNA, whereas expression is low in neonatal buccal ECs.

Figure 2 Priming of naive TR146 cells with peptidoglycan results in hyporesponsiveness that is associated with the expression of SLPI. (A) TR146 cells were cultured in triplicate for 24 h with or without peptidoglycan (PG, 50 $\mu\text{g}/\text{mL}$), supernatant was collected and the cells were washed. Next, the cells were restimulated with or without PG (50 $\mu\text{g}/\text{mL}$) for 24 h. CXCL-8 production was determined in the supernatant at $t=24$ h and at $t=48$ h. Data are means with SEM ($n=3$). p Values were calculated using an unpaired t test. Data are representative of 3 independent experiments. (B) TR146 cells were cultured in duplicate for 24 h with or without PG (50 $\mu\text{g}/\text{mL}$). A proportion of the cells was collected after 24 h of culture, and the remaining cells were then stimulated with PG for 5, 15 and 45 minutes. Phosphorylated NF- κB protein bands were analysed by western blot in the nuclear fractions of cellular lysates. Within the cytosolic fraction of cellular lysates, total I $\kappa\text{B}\alpha$ protein bands and phosphorylated I $\kappa\text{B}\alpha$ protein bands were analysed by western blot. Densitometry of the bands was used to determine NF- κB and I $\kappa\text{B}\alpha$ levels relative to ORC2 (nuclear) or β -actin (cytosolic), and these were expressed as arbitrary units (AU). At $t=0$ min, AU were set at 0. Data are representative of two independent experiments. (C) TR146 cells were stimulated in duplicate for 24 h with PG (50 $\mu\text{g}/\text{mL}$) or left unstimulated, and cells were harvested for SLPI mRNA analysis by quantitative SYBR Green PCR. Data are expressed as means with SEM. p Values were calculated using an unpaired t test. (D) TR146 cells were cultured in triplicate for 24 h with or without PG (50 $\mu\text{g}/\text{mL}$). Supernatant was collected for detection of SLPI protein using ELISA. Data are representative of four independent experiments and expressed as means with SEM ($n=3$). p Values were calculated using an unpaired t test. (E) TR146 buccal epithelial cells were cultured with 50 $\mu\text{g}/\text{mL}$ peptidoglycan (PG) or without stimulus for 24 h, after which cellular lysates from duplicate wells were analysed for A20, Tollip, SIGIRR and SLPI mRNA expression by quantitative SYBR Green PCR. Data are expressed as means with SEM. p Values were calculated using an unpaired t test.



SLPI regulates acquisition and maintenance of hyporesponsiveness in intestinal epithelium.

Since TR146 ECs and Caco-2 ECs acquired an SLPI-driven hyporesponsive state after continued exposure to TLR2 stimulation, we reasoned that the epithelium throughout the gastrointestinal tract may use similar mechanisms to acquire a hyporesponsive state. Therefore, we first examined the

induction of SLPI expression and the mouse homologue of CXCL-8 (CXCL-2) in the intestine of adult germ-free mice which were colonised with conventional microflora over time.²² Comparative transcriptome analyses were performed on small intestinal and colonic tissue of germ-free and conventionalised mice sacrificed on day 0, day 4 and day 30 after colonisation. Samples were also collected for immunohistochemical analysis.

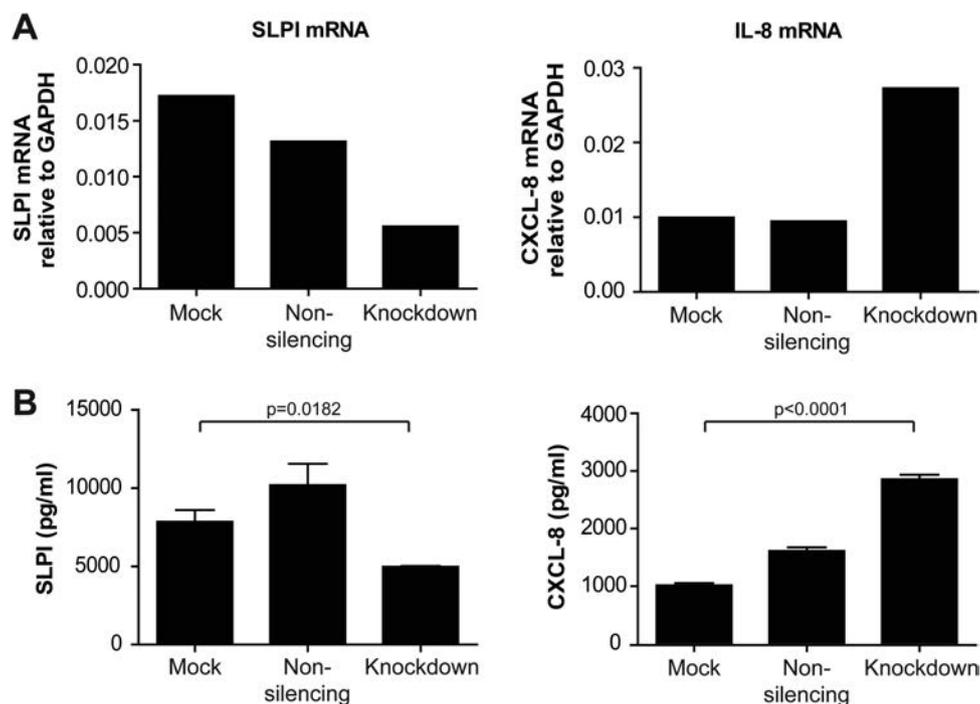


Figure 3 Knockdown of SLPI in the buccal epithelial cell line TR146 results in spontaneous high CXCL-8 production. (A) TR146 cells were transduced with virus supernatant containing an empty vector (mock), shRNA for a non-silencing control and shRNA specific for SLPI. The transduced TR146 cells were cultured in the presence of 1 μ g/mL puromycin for 14 days. After stable culture for 21 days the cells were collected for SLPI and CXCL-8 mRNA analysis by quantitative SYBR Green PCR. Data are expressed as means (n=2). (B) TR146 cells were cultured for 24 h without stimulus, SLPI and CXCL-8 protein in supernatant was detected by ELISA. Data are expressed as means with SEM (n=3). p Values were calculated using an unpaired t test.

In the small intestine, SLPI gene expression was slightly higher at day 30 of colonisation compared to day 0 (figure 5A). In the colon, induction of SLPI gene expression was more pronounced and significantly increased on day 4 after conventionalisation, and expression remained high on day 30 (figure 5A). In agreement, immunohistochemistry revealed abundant SLPI protein in colonic epithelium at day 4 and day 30 of colonisation (figure 5B). In the small intestine, SLPI protein was less abundant than in the colon. These data argue that bacterial colonisation induces increased SLPI expression in the intestine. To assess a possible correlation between SLPI expression and epithelial hyporesponsiveness CXCL-2 expression was determined. Although CXCL-2 gene expression was significantly increased on day 4 after conventionalisation in small intestine and colon, the expression returned to germ-free level on day 30 in the colon and tended to decrease in the small intestinal ileum (figure 5A, B). Collectively, these data show that colonisation of the colon results in increased SLPI and CXCL-2 expression at day 4. However, during extended colonisation, an inverse relationship between SLPI and CXCL-2 expression developed. To formally demonstrate that SLPI expression is needed for inhibition of chemokine release by intestinal ECs in vivo, we examined CXCL-2 and CXCL-9 expression in freshly isolated intestinal ECs of normally (SPF) colonised SLPI-deficient mice. Indeed, small intestinal ECs of SLPI-deficient mice expressed significantly higher mRNA levels of CXCL-2 and CXCL-9 compared to SLPI-sufficient mice (figure 6). In colonic epithelium of SLPI-deficient mice, the increase of CXCL-2 and CXCL-9 mRNA showed the same trend but did not reach significance, however, immunohistochemistry confirmed increased CXCL-2 protein levels in colonic ECs of SLPI-deficient animals (figure 6).

DISCUSSION

In this study, we have shown that primary buccal ECs secrete CXCL-8 at birth, and that this secretion is enhanced in response to microbial triggering. In the first weeks of life, ECs actively acquired hyporesponsiveness to microbial stimulation. Hyporesponsiveness of ECs corresponded with a decrease in I κ B degradation and a subsequent decrease in NF- κ B activation, and also with an upregulation of SLPI. We show that SLPI is a crucial mediator for acquisition of epithelial hyporesponsiveness, as knockdown of SLPI expression in buccal ECs resulted in increased CXCL-8 production. This function of SLPI was shared between buccal epithelium and colonic epithelium, as colonisation of the intestine of germ-free mice induced persistent upregulation of SLPI expression in small intestinal and colonic epithelium after introduction of the microbiota. Upon extended colonisation, this upregulation of SLPI was associated with decreased CXCL-2 production by colonic epithelium. Furthermore, small intestinal and colonic ECs of SLPI-deficient mice spontaneously exhibited enhanced CXCL-2 mRNA expression compared with SLPI-sufficient mice.

Using human primary buccal ECs, we show that the transition from the sterile in utero environment to initial and persistent contact with microbial products after birth is a pivotal event in shaping human epithelial responses. Within minutes after birth, buccal ECs from neonates were found to release CXCL-8 which was increased by microbial triggering. This epithelial responsiveness to bacterial stimulation is not restricted to the buccal cavity, as several groups have shown responsiveness of neonatal intestinal epithelium to pathogen-associated molecular patterns (PAMPs).^{6 23 24} The accessibility of the buccal epithelium allowed us to follow epithelial cell function in the first weeks of life within individual subjects. Despite variation in initial

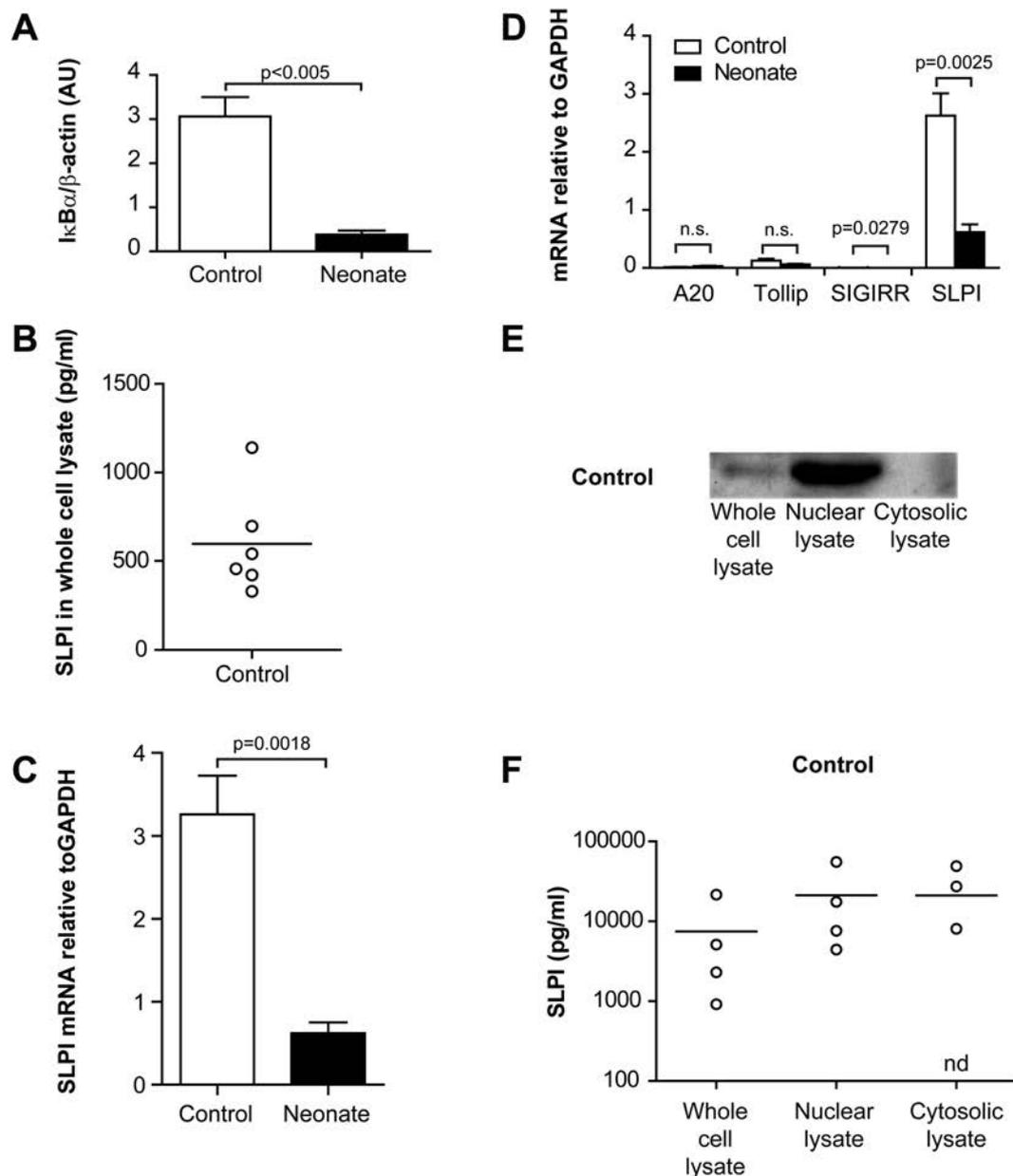


Figure 4 Neonatal buccal epithelial cell sensitivity to microbial stimulation is associated with decreased SLPI expression. (A) Buccal epithelial cells (EC) from controls (age 29–55 years) and neonates were collected, lysed and analysed by western blot for IκBα and β-actin proteins. Densitometry of IκBα protein bands was used to determine IκBα levels. Levels were corrected for β-actin and denoted as arbitrary units (AU). p Values were calculated using unpaired sample t test with Welch correction. (B) Buccal ECs from 6 controls (ages 29–55 years) were collected, and SLPI expression was assessed by ELISA in whole cell lysate. (C) Buccal ECs from controls (n=5, age 29–55 years) and neonates (n=4, age <24 h) were collected and directly lysed for analysis of SLPI mRNA expression. Data are expressed as means with SEM. p Values were calculated using an unpaired t test. (D) Buccal ECs from 4 controls (ages 29–55 years) and 4 neonates (aged less than a day old) were analysed for A20, Tollip, SIGIRR and SLPI mRNA expression by quantitative SYBR Green PCR. Data are expressed as means with SEM. p Values were calculated using an unpaired t test. (E) Buccal ECs from controls (age 29–55 years) were collected and incubated without stimulus for 24 h. Cells were lysed, nuclear and cytosolic fractions isolated, and western blot was performed to detect SLPI protein. (F) Buccal ECs from controls (age 29–55 years) were collected and incubated without stimulus for 24 h. Afterwards cells were lysed, nuclear and cytosolic fractions isolated, and SLPI protein was detected by ELISA. n.d. denotes non-detectable.

responsiveness, buccal ECs of primary caesarean section-delivered neonates gradually acquired microbial hypo-responsiveness within the first 3 weeks of life. In analogy, it was previously shown in a murine model that the first encounter to endotoxin postpartum elicits chemokine production by intestinal epithelium.⁶ However, 24 h after birth the exposure to exogenous LPS had rendered the murine intestinal ECs unresponsive towards endotoxin. In our study, primary buccal ECs acquired

hypo-responsiveness within a minimum of a few days after birth, indicating that a responsive period precedes the tolerant state of buccal ECs found in healthy individuals. Hypo-responsiveness of adult buccal ECs was not due to death or anergy, as buccal ECs were viable and showed calcium fluxes (see online supplementary figure 1). Moreover, TLR2 mRNA expression was found to be similar or higher in hypo-responsive adult buccal ECs compared to neonatal buccal ECs (see online supplementary figure

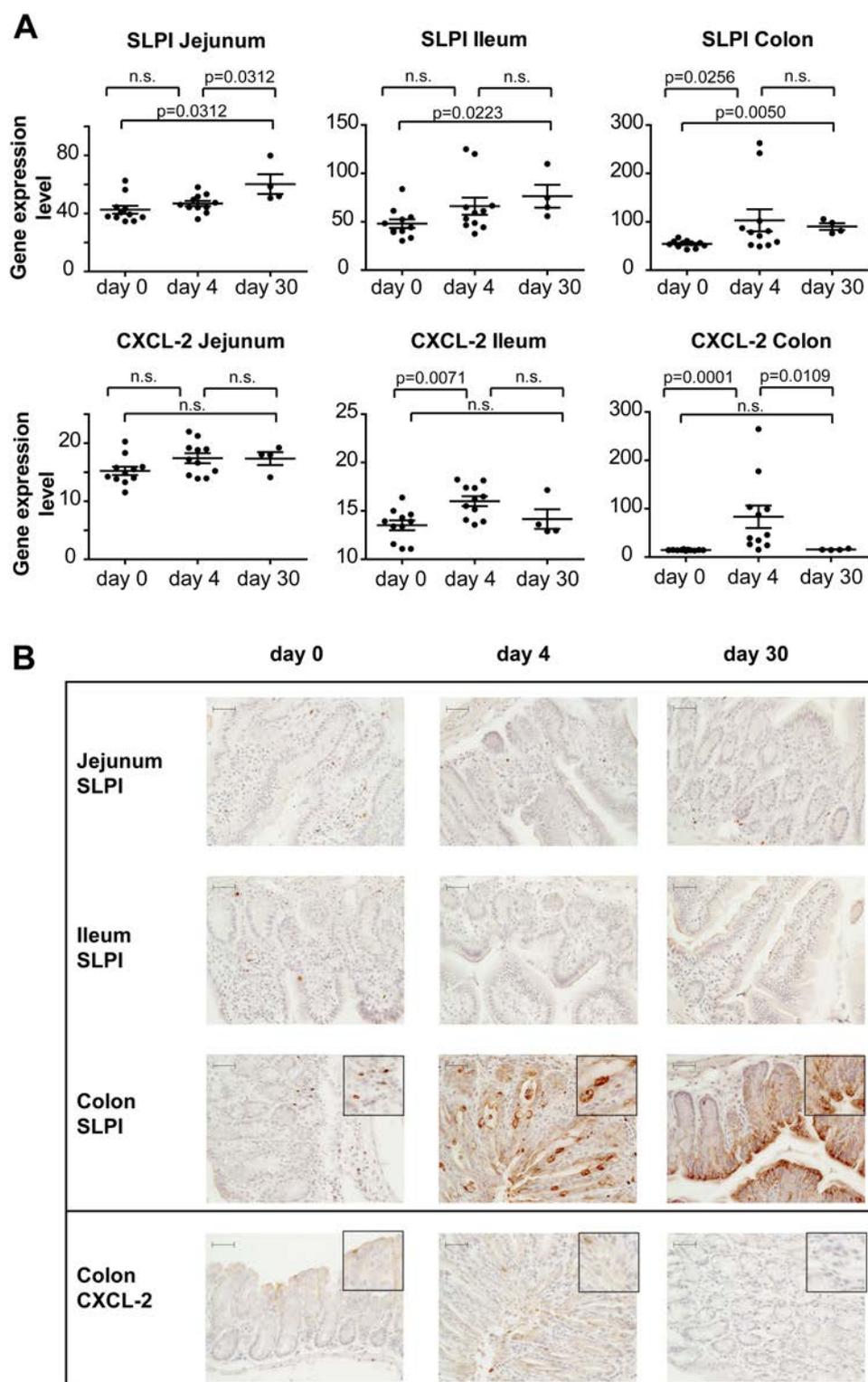


Figure 5 Colonisation of the intestine of germ-free (GF) mice results in increased SLPI and CXCL-2 expression in the colon. C57BL/6 mice were bred under GF conditions and colonised with conventional microbiota. Mice were sacrificed on day 0, day 4 and day 30 after conventionalisation, and samples of jejunum, ileum and colon were taken for mRNA microarray analysis or embedded in paraffin for immunohistochemical analysis. (A) SLPI and CXCL-2 gene expression in intestinal segments of conventionalised mice. Dot plots represent the expression levels of SLPI and CXCL-2 in the intestinal tissues. p Values were calculated using a Mann–Whitney analysis; n=4–11 for each time-point. (B) Immunohistochemical SLPI and CXCL-2 staining on paraffin embedded sections of intestinal tissue from conventionalised mice. Data are representative of three mice. Scale bar indicates a magnification of 40 \times .

1). Therefore, although we cannot fully rule out that reduced TLR surface expression or subcellular localisation plays a role in buccal ECs hypo-responsiveness, we explored whether repetitive exposure of naive ECs to microbial ligands leads to the

production of proteins that regulate the NF- κ B pathway. Active induction of hypo-responsiveness to microbial stimulation has been described in multiple intestinal cell lines.¹⁷ Indeed, cells from the buccal epithelial cell line TR146 as well as the colonic

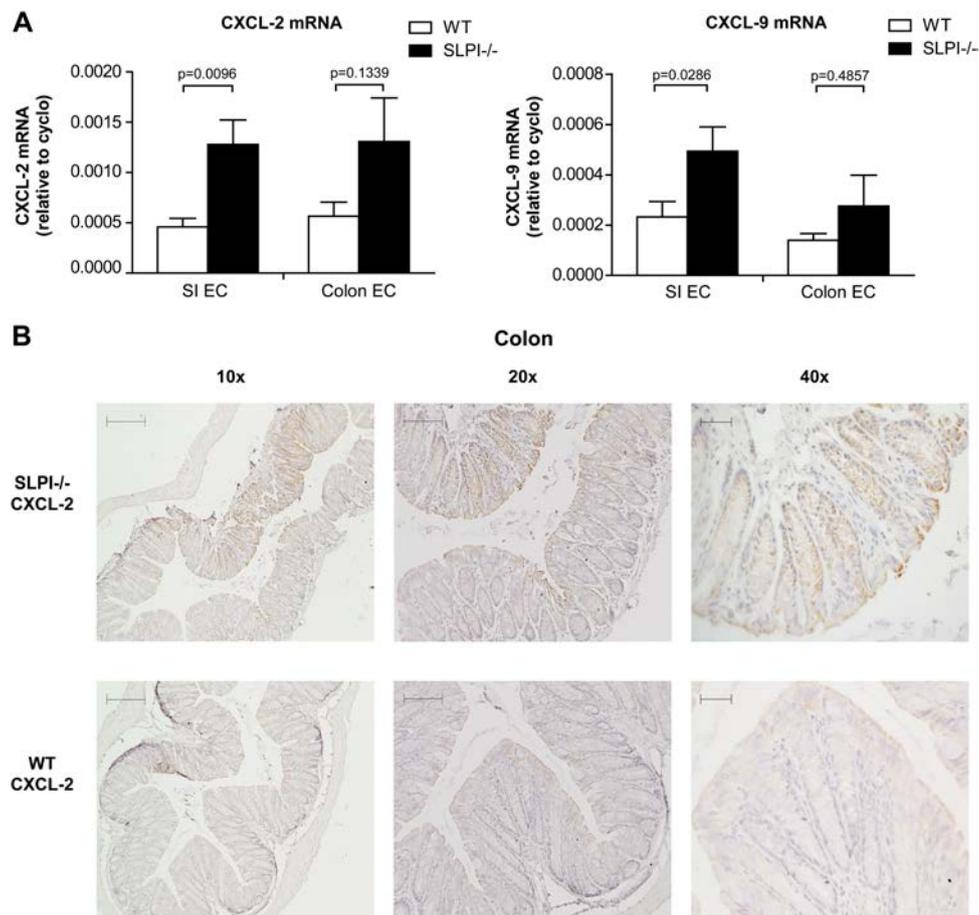


Figure 6 CXCL-2 and CXCL-9 expression is spontaneously enhanced in the intestinal epithelium of SLPI-deficient mice compared to WT mice. (A) C57BL/6 WT and SLPI-deficient mice were sacrificed and small intestinal and colonic epithelial cells were isolated for mRNA analysis. CXCL-2 and CXCL-9 mRNA expression was assessed by quantitative SYBR Green PCR. Data are representative of two independent experiments. Data are expressed as mean with SEM (n=4 mice per group). p Values were calculated using an unpaired t test for the CXCL-2 data, and a Mann-Whitney analysis for the CXCL-9 data. (B) C57BL/6 WT and SLPI-deficient mice were sacrificed, and distal colon was embedded in paraffin and stained for CXCL-2 by immunohistochemistry. Data are representative of four C57BL/6 and four SLPI-deficient mice.

epithelial cell line Caco-2 pretreated for 24 h with a TLR ligand showed decreased CXCL-8 production in response to restimulation with the same ligand. Hyporesponsiveness of the ECs was associated with reduced NF- κ B activation. However, the Caco-2 cells which had become hyporesponsive to Pam3Cys were still able to respond to high-dose IL-1 β stimulation to the same extent as responsive cells, indicating that the cells were not defective in NF- κ B signalling (data not shown). Using this model system we searched for molecular pathways that are involved in the hyporesponsiveness of ECs. Microbial-associated molecular patterns are known to have the capacity to induce regulatory molecules. As such, IRAK-M, NOD2, Tollip, A20, SIGIRR and SLPI have been found to inhibit intracellular signal transduction at various stages during the signalling cascade, ultimately leading to reduced NF- κ B activation and inflammatory gene expression.⁸⁻¹³ In our experiments, we identified that microbial hyporesponsiveness in TR146, Caco-2 cells and primary adult buccal ECs were associated with upregulated SLPI mRNA expression and SLPI protein, while other regulatory molecules were expressed at low levels or were undetectable. Subsequent knockdown of SLPI in the TR146 cells revealed that SLPI constitutively suppresses CXCL-8, CXCL-9 and CXCL-10 production in buccal ECs. It should be noted that in contrast with human buccal ECs, fresh murine adult and neonatal cheek epithelium releases substantial amounts of chemokines at

baseline. However, the epithelium is hyporesponsive to a further stimulation with microbial PG. SLPI did not mediate this hyporesponsiveness (see online supplementary figure 4). As high levels of the NF- κ B inhibitor Tollip are expressed by murine cheek epithelium, we anticipate that Tollip is the major NF- κ B regulator maintaining homeostasis in murine cheek epithelium (see online supplementary figure 4).

SLPI is a potent inhibitor of the NF- κ B pathway that interferes with microbial-induced TLR activation at multiple levels. In particular, the secretory form of SLPI can directly interact with LPS before the latter molecule interacts with responder cells.¹⁹ Intracellular SLPI can inhibit Toll-like receptor 2 and 4 signalling by directly preventing the degradation of the inhibitory factor I κ B α .²⁰ Additionally, Taggart *et al* demonstrated that SLPI translocates to the nucleus directly binding to the NF- κ B consensus sequence in the promoter region of the CXCL-8 and TNF- α genes.¹¹ In particular, when incubating monocytic U937 cells or peripheral blood monocytes for 1 h with exogenous labelled SLPI, the labelled protein could be found in the cytosol and nucleus of these cells.¹¹ SLPI bound to wild-type biotinylated NF- κ B consensus oligonucleotide in a dose-dependent manner.¹¹ A single base change in the biotinylated consensus sequence of NF- κ B rendered SLPI unable to bind.¹¹ As such, SLPI may compete with the NF- κ B p65 subunit for binding to this consensus sequence, thus blocking transcription. In TR146

cells, at least the latter two of these mechanisms play a role in SLPI-mediated epithelial unresponsiveness. As such, in hyporesponsive cells, cytoplasmic levels of I κ B were enhanced in the presence of SLPI (figure 2B), and nuclear SLPI could clearly be detected (see online supplementary figure 2).

In view of its potent function in TR146 cells, SLPI expression was also investigated in primary buccal ECs. Strikingly, hyporesponsive primary buccal ECs of adult individuals expressed significantly higher levels of SLPI mRNA compared to responsive neonatal buccal ECs. Furthermore, in hyporesponsive buccal ECs, SLPI protein was not only found in the cytosol, but was also detectable in the nucleus at very high concentrations. Future study is required to assess whether, through its nuclear localisation, SLPI inhibits inflammation directly by binding the NF- κ B consensus sequence in the promoter region of the CXCL-8 gene.¹¹ Although SLPI is a known regulator of the NF- κ B pathway, its role in driving EC hyporesponsiveness has been underscored. Besides high expression of SLPI in mucosal ECs, it is also produced in mucosal secretions such as saliva and tears. As such, it has potent antiprotease activity providing a first barrier of protection against microbial proteases.²⁵

In previous work, we demonstrated that buccal ECs of paediatric patients with Crohn's disease without oral lesions are responsive to microbial stimuli, while healthy controls were hyporesponsive.¹⁵ This raises the question whether a particular mechanism accounts for the loss of hyporesponsiveness in the epithelium of paediatric patients with Crohn's disease. Recently, in murine experiments SLPI was implicated in recovery from colonic inflammation.²⁶ Here, we have shown that SLPI suppresses chemokine production in buccal and intestinal epithelium. Within the intestine, the inhibitory role of SLPI in regulating the epithelium is evident in the small intestine and colon. In the small intestine, SLPI expression was more dominantly detected in cells in the lamina propria, whereas in the colon, SLPI expression was most evident within the epithelium. SLPI-deficient mice exhibited spontaneous CXCL-2 expression by intestinal epithelium, however, this did not lead to the development of colitis in these mice. Our data show that SLPI is a crucial mediator in the acquisition and maintenance of epithelial hyporesponsiveness by directly limiting NF- κ B activation. Therefore, these results warrant further study to unravel the possible role of altered SLPI-mediated NF- κ B inhibition in chronic inflammation in the intestine.

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Human buccal epithelium acquires microbial hyporesponsiveness at birth, a role for secretory leukocyte protease inhibitor

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