

Giardia lamblia-Induced Changes in Gene Expression in Differentiated Caco-2 Human Intestinal Epithelial Cells†

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Received 31 May 2005/Returned for modification 31 July 2005/Accepted 22 September 2005

The parasitic protozoan *Giardia lamblia* is a worldwide cause of diarrhea, but the mechanism of disease remains elusive. The parasite colonizes the small intestinal epithelium, known to be a sensor for the presence of enteric pathogens, without invading or causing severe inflammation. In this study we investigated the epithelial cell response to *G. lamblia*. Differentiated Caco-2 cells were infected with *G. lamblia* isolate WB-A11, and the transcriptome of the intestinal cells was analyzed after 1.5, 6, and 18 h of interaction, using oligonucleotide microarrays. A large number of genes displayed changed expression patterns, showing the complexity of the interaction between *G. lamblia* and intestinal cells. A novel chemokine profile (CCL2, CCL20, CXCL1, CXCL2, and CXCL3) was induced that was different from the response induced by enteric pathogens causing intestinal inflammation. Several genes involved in stress regulation changed their expression. These findings indicate that the intestinal epithelium senses the *G. lamblia* infection, and this is important for induction of innate and adaptive immunity. The induced stress response can be important in the pathogenesis.

Giardia lamblia is a leading cause of waterborne diarrhea in humans and other mammals (1). Infection begins with the ingestion of cysts followed by the excysted trophozoites colonizing the host's small intestine. The clinical spectrum varies from asymptomatic carriage to chronic infection and malabsorption (13). The disease mechanism is enigmatic, with suggested causes ranging from disturbance of intestinal enzymes and damaged mucosa to increased intestinal permeability and disturbance of the normal flora (13, 27, 28).

Infection with *G. lamblia* is often self limiting, indicating the presence of an effective host defense (11). The immune mediators must act locally in the lumen, since *G. lamblia* does not invade the epithelial layer, and there is only slight mucosal inflammation (23). Secretory immunoglobulin A (sIgA) antibodies play a central role in the clearance of the parasite (11, 18) along with other host factors, such as nitric oxide (NO) (12), defensins (2), and mast cells (19).

To further understand the host defense and elucidate the pathogenicity of *G. lamblia*, it is important to characterize the host-parasite interaction. Previously, no human host genes have been identified showing gene expression changes due to infection of *G. lamblia*, whereas interleukin-6 (IL-6) expression is induced during experimental mouse infections (30). In this study, the transcriptional response of *G. lamblia*-infected human intestinal epithelial cells is analyzed using Affymetrix oligonucleotide microarrays.

MATERIALS AND METHODS

Cell culture and colonization of enterocytes. *G. lamblia* trophozoites (WB clone A11 and GS/M-83-H7) were cultivated in TYI-S-33 medium supplemented with bile according to Keister (17). Caco-2 cells, clone TC7, were obtained from

Monique Rousset and grown according to Chantret et al. (6). The cells were allowed to differentiate into enterocytes by seeding at a density of 2×10^4 cells/cm² in 6-well dishes and culturing them for 21 days, changing the medium twice each week. Cells were also grown on Transwell filters (0.4 μ m pore size). The differentiated phenotype was confirmed by a higher mRNA expression of intestinal alkaline phosphatase and aminopeptidase N compared to that of non-differentiated cells. The presence of tight junctions was confirmed by zonula occludens 1 (ZO-1) immunofluorescence (data not shown). All cells were mycoplasma free.

Exponentially growing trophozoites were harvested by chilling for 20 min in ice, collected by centrifugation (5 min at 2,000 rpm, 4°C), washed twice with phosphate-buffered saline (PBS), and resuspended in Dulbecco's modified Eagle medium before adding to the Caco-2 cells. Trophozoites (1×10^7 /well) were added to differentiated Caco-2 cells, which had been replenished with fresh medium 1 to 2 h before the experiment, and incubated for 1.5 to 18 h at 37°C. Control (unstimulated) enterocytes had no parasites added. The culture medium was collected after coinubation (1.5, 6, or 18 h) and stored at -20°C. Trophozoites were detached and removed by rinsing the intestinal cells four times with 4°C PBS. Intestinal cells were collected in 4°C PBS using a cell scraper and centrifugation.

Sample preparation and array hybridization. For each time point (1.5, 6, and 18 h), total RNA was isolated from two independent experiments of unstimulated and *G. lamblia*-infected Caco-2 cells using the RNeasy kit (QIAGEN, Stanford, CA). The integrity of the RNA was analyzed in a 2100 Bioanalyser (Agilent Technologies Inc., Palo Alto, CA). The RNA was processed, labeled, and hybridized to the Affymetrix GeneChips Human Genome Focus Array in the Affymetrix core facility at NOVUM (Karolinska Institutet) according to the GeneChip Expression Analysis technical manual (no. 701021, revision 1). The probed arrays were scanned in the Affymetrix scanner, and Affymetrix Microarray Suite (MAS) 5.1 software was used for calculation of absolute and comparison data.

Reverse transcription-PCR (RT-PCR) analysis. Semiquantitative reverse transcription (RT) was carried out using an oligo(dT) primer, SuperScript II RNase H⁻ Reverse Transcriptase (Invitrogen, Carlsbad, CA), and 1 μ g total RNA from at least three independent experiments. The resulting cDNA was amplified using PureTaq PCR beads (Amersham Biosciences, Piscataway, NJ) and primers spanning over at least one intron (supplementary material S4). PCRs were optimized to be in the linear amplification range and, depending on primer sets, were run in 20 to 25 amplification cycles of denaturation (96°C for 30 s), annealing (56 to 60°C for 30 s), and extension (72°C for 1 min), followed by a final extension (72°C for 7 min). The PCR products were visualized by agarose gel electrophoresis, and GAPDH was used as an endogenous control.

To further validate the gene expression changes, quantitative real-time RT-PCR analysis was performed using an Applied Biosystems Prism 7900HT Se-

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† Supplemental material for this article may be found at <http://iai.asm.org>.

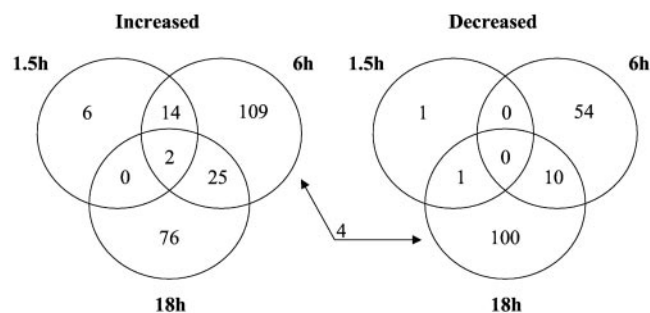


FIG. 1. Number of genes regulated upon *G. lamblia* interaction. Distribution of genes with significantly altered gene expression of twofold or more according to time. Commonly, the v-fos FBJ murine osteosarcoma viral oncogene homologue (FOS) and the epithelial-specific ets domain transcription factor, E74-like factor 3 (ELF3), were induced at all time points. Four genes were induced at 6 h and then repressed after 18 h (regulator of G protein signaling [RGS2], v-myc myelocytomatosis viral oncogene homologue [avian, MYC], inhibitor of DNA binding 1 [ID1], and pleckstrin homology-like domain, family A, member 2 [PHLDA2]).

quence Detection System according to manufacturer's specifications (Foster City, CA). Human cDNA-specific TaqMan Gene Expression Assays for CCL20 (assay identification number Hs00171125_m1), inducible nitric oxide (iNOS) (assay identification number Hs00167248_m1), and NOX1 (assay identification number Hs00246589_m1) from Applied Biosystems were used in the study. A human 18S rRNA gene was used as an endogenous control (product number 4319413E; Applied Biosystems). The gene-specific minor groove binder probes were 6-carboxyfluorescein labeled, and the 18S rRNA-specific minor groove binder probe was VIC (fluorescent reporter dye; Applied Biosystems, Rockville, Md.) labeled and primer limited. The thermal cycler conditions were as follows: initiation with a "hot-start" (95°C for 10 min) followed by a two-step PCR for 40 cycles (95°C for 15 s followed by 60°C for 1 min). All samples were run in triplicate, and amplification data were analyzed using Applied Biosystems Prism Sequence Detection Software, version 2.2.1. Relative quantification was calculated according to the $\Delta\Delta C_t$ method (Applied Biosystems Inc., Foster City, CA) using a statistical confidence of 99.9%.

Enzyme-linked immunosorbent assay (ELISA). The quantification of chemokine CCL20 (MIP-3 α) secretion was determined by a Human MIP-3 α Immunoassay (R&D Systems, Wiesbaden, Germany). The amount of CCL20 was quantified in triplicate using supernatants from three different experiments.

RESULTS AND DISCUSSION

Genome data analysis. The topmost single layer of intestinal epithelial cells is an integral and essential component of the mucosal immune system. In this study, we analyzed the gene expression in human intestinal epithelial cells (differentiated Caco-2 cells) incubated with *G. lamblia* (WB-A11) trophozoites. After 1.5, 6, and 18 h coincubation, total RNA was prepared from infected and uninfected Caco-2 cells and hybridized to microarrays containing 8,746 probe sets. Almost half (48%) of the genes represented on the microarray were expressed in Caco-2 cells at all time points (supplemental material S1). For each time point, duplicates of infected cells were compared to duplicates of uninfected cells in a 2 by 2 matrix (supplemental material S2). Signal values from all duplicates showed a correlation coefficient of >0.99. The genes that were significantly increased or decreased ($P < 0.0045$; Wilcoxon's signed-ranks test) in all four comparisons with an average change of at least twofold were selected for further analysis (supplemental material S3).

During the experiment, 236 genes increased and 170 genes decreased their expression more than twofold (Fig. 1). Two genes were increased in all time points, and four genes were increased at 6 h and then repressed after 18 h (Fig. 1). The extensive changes in gene expression are reflected in the large number of up- and down-regulated transcriptional regulators (supplemental material S3). Early induced genes (1.5 h) are predominantly associated with the immune signaling (Fig. 1, Table 1). After 6 h of interaction, genes involved in proliferation, stress, and/or hypoxic responses changed their expression, being even more pronounced after 18 h. The down-regulated transcriptome at 18 h (Fig. 1) indicates that the epithelial cells are being affected by the *G. lamblia* colonization, possibly due to competition for nutrients, as a direct result of the parasite, or both. The expression patterns of 10 genes with different expression profiles on the microarrays were confirmed using semiquantitative (Fig. 2) and real-time RT-PCR (supplemental material S4).

TABLE 1. Gene expression and change in induction

Function	Designation	Description	Acc. no. ^a	Fold change ^b at:		
				1.5 h	6 h	18 h
CC chemokines	CCL2	Chemokine (C-C motif) ligand 2	S69738	14.9		
	CCL20	Chemokine (C-C motif) ligand 20	NM_004591	67.4	142	
CXC chemokines	CXCL1	Chemokine (C-X-C motif) ligand 1	NM_001511	10.6		
	CXCL2	Chemokine (C-X-C motif) ligand 2	M57731	24.3	2.9	
	CXCL3	Chemokine (C-X-C motif) ligand 3	NM_002090	19.0		
Other chemoattractant Receptors	PLAUR	Plasminogen activator, urokinase receptor	U08839		7.6	2.9
	CXCR4	Chemokine (C-X-C motif) receptor 4	AJ224869		11.7	2.9
	ICAM1	Intercellular adhesion molecule 1 (CD54)	NM_000201		2.5	
Transcription regulators	ELF3	E74-like factor 3 (ets domain transcription factor, epithelial specific)	U73844	3.0	3.7	2.3
	FOS	v-fos FBJ murine osteosarcoma viral oncogene homologue	BC004490	7.3	6.0	2.8
	JUNB	jun B proto-oncogene	NM_002229	2.7	5.3	
	NFKBIA (I κ B α)	Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha	AI078167	8.0	5.3	
Regulators of cell cycle	PCNA	Proliferating cell nuclear antigen	NM_002592		0.47	0.33
	G0S2	Putative lymphocyte G $_0$ /G $_1$ switch gene	NM_015714		0.55	0.15

^a NCBI GenBank accession number.

^b For each gene, the average ratios from four comparisons are reported.

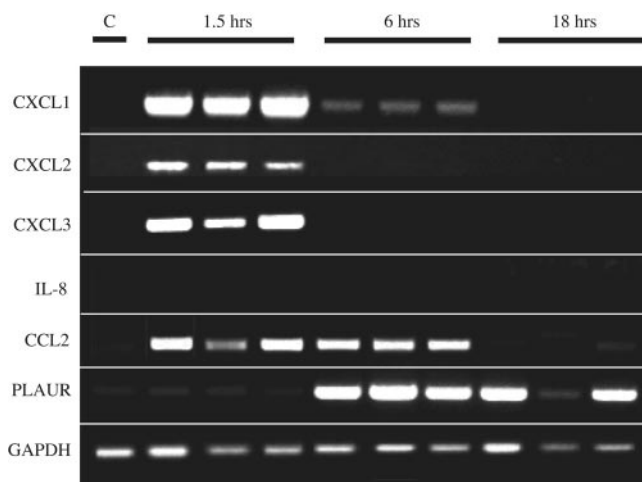


FIG. 2. Semiquantitative RT-PCR analysis of chemokine expression. Total RNA was isolated from *G. lamblia*-infected or uninfected Caco-2 cells after 1.5, 6, and 18 h of interaction and analyzed by semiquantitative RT-PCR using primers specific for CXCL1, CXCL2, CXCL3, IL-8, CCL2, PLAUR, and GAPDH. The results from three independent experiments are shown. The control levels were constant throughout the experiment, and one lane corresponding to the 1.5-h control (C) is shown.

Chemokine response in host cells to *G. lamblia* infection. In response to pathogens, intestinal enterocytes produce proinflammatory chemokines and cytokines that regulate the immune response (20). We detected a strong chemokine response after 1.5 h of infection, with up-regulation of CC chemokine ligand 20 (CCL20), CCL2, CXCL1, CXCL2, and CXCL3 (Table 1). This is a unique chemokine profile, and it indicates a recruitment of dendritic cells (DCs), T cells, and B cells by CCL20, neutrophils by CXCL1 to CXCL3, and macrophages and T-cells by CCL2 to the site of infection. The up-regulation of all five chemokines early during the interaction was confirmed by semiquantitative (Fig. 2) and real-time RT-PCR (supplemental material S4). Addition of parasite growth medium did not induce the expression of the chemokines (data not shown).

CCL20 dominated the response, and after 6 h it was increased 142-fold (Table 1). The CXCL chemokines were downregulated earlier than the CCL chemokines, and after 6 h they were only weakly expressed (Fig. 2). In addition, the chemokine receptor CXCR4, the tumor necrosis factor alpha (TNF- α) receptor TNFRSF1A, and the adhesion molecule ICAM-1 were induced at 6 h (Table 1), indicating an activation of the intestinal epithelium to respond to chemokines and cytokines and promote leukocyte binding. The array analysis and RT-PCR analysis (Fig. 2) showed an upregulation of the plasminogen activator urokinase receptor (PLAUR) after 6 h, and in a soluble form it acted as a chemoattractant (3).

The proinflammatory cytokines interleukin-1 (IL-1), IL-6, IL-8, and TNF- α and the immunoregulatory cytokines IL-2, gamma interferon (IFN- γ), and IL-10 were not induced, consistent with earlier results (16). The expression of IL-8 was specifically investigated using semiquantitative RT-PCR (Fig. 2), and it was very weakly expressed, but a low level of upregulation could be detected after a long exposure of the gel (data

not shown). This is in contrast to enteric bacteria, viruses, and other protozoans, where IL-8 is the key cytokine inducing inflammation (8, 10, 24), but in agreement with the observation that *G. lamblia* causes little or no inflammation in humans (23). Nuclear factor- κ B (NF- κ B) is a key regulator of immune genes, including those encoding chemokines. In our experiment, the NF- κ B inhibitors I κ B α and Bcl-3 were strongly induced at 6 h, suggesting that NF- κ B is important early during the *G. lamblia* colonization (Table 1). Similarly, I κ B α was induced in *Shigella flexneri*-infected Caco-2 cells (24) and in *S. flexneri*- and *Entamoeba histolytica*-infected human colonic xenografts (29). Although these responses indicate a general involvement of NF- κ B during enteric infections, the expression of NF- κ B-regulated genes differs between the different organisms and demonstrates the importance of other transcription factors in achieving specificity in the response.

CCL20 expression. According to our microarray data, the strongest *G. lamblia*-induced gene was CCL20, and this is interesting since it can be important for processing of *Giardia* antigens by DCs (5). With semiquantitative RT-PCR, we could show that the level of CCL20 induction was cell density dependent (Fig. 3A). The upregulation of CCL20 was verified using real-time RT-PCR, and the level of CCL20 mRNA was upregulated approximately 40- and 90-fold after 1.5 and 6 h of interaction, respectively (supplemental material S4). A similar response was seen when using another *G. lamblia* isolate, GS/M-83-H7, and also when infecting HT29 intestinal cells (data not shown). In order to determine if the induced CCL20 is translated and secreted apically or basolaterally, *G. lamblia* trophozoites were added on the apical side of polarized differentiated Caco-2 cells, and the CCL20 production was monitored by ELISA. As shown in Fig. 3B, CCL20 is produced and secreted dominantly basolaterally. To be noted, the increased apical production of CCL20 in naïve Caco-2 cells at 18 h does not correspond to CCL20 mRNA levels, but it is possible that this CCL20 is released from intracellular storage.

In an effort to dissect the mechanism by which the *G. lamblia* colonization induces an increase in CCL20, trophozoites were physically separated from the differentiated Caco-2 cells with a 0.4- μ m pore Transwell filter and the CCL20 production was monitored. CCL20 secretion was induced without *Giardia* being able to attach to the Caco-2 cells (Fig. 2C), indicating that this chemokine activation is probably caused by a secreted parasite factor(s). The same result was observed when differentiated Caco-2 cells were allowed to interact with trophozoite-free medium from a *G. lamblia* culture (data not shown).

CCL20 is thought to be the major chemokine in guiding immature DCs from the blood to intestinal tissues. In the lamina propria, DCs present antigen to the adaptive immune system, leading to the production and release of sIgA (5). We speculate that the *G. lamblia*-induced basolateral secretion of CCL20 recruits DCs and/or activates DCs beneath the epithelium, and subsequently this results in the secretion of *Giardia*-specific sIgA.

To summarize the chemokine-cytokine section, we conclude that *Giardia* can induce the expression of CXCL1 to CXCL3, CCL2, and CCL20 in differentiated Caco-2 cells. Interestingly, CXCL1 to CXCL3 and IL-8 are upregulated in biopsies from patients with active colonic inflammatory bowel disease (26). *Giardiasis* has been shown to be a triggering factor of irritable

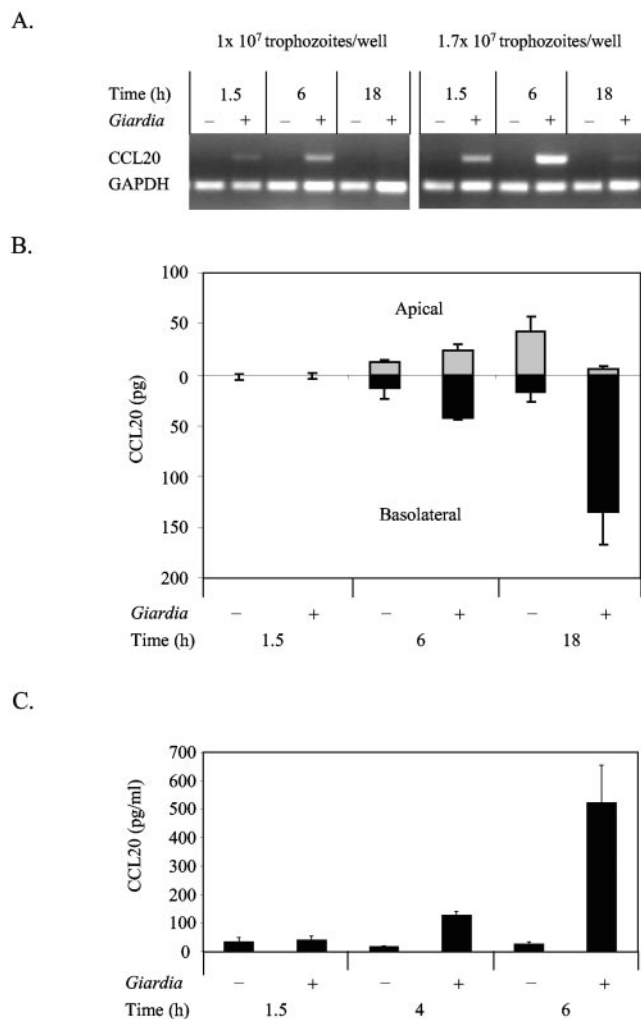


FIG. 3. Expression of CCL20. A) Dosage-dependent gene expression of CCL20. After 1.5, 6, and 18 h, total RNA was isolated from *G. lamblia*-infected or uninfected Caco-2 cells and analyzed by RT-PCR using CCL20- or GAPDH-specific exon-spanning primers. One representative result from three independent experiments is shown. B) Secretion of CCL20 of polarized and differentiated Caco-2 cells. Caco-2 cells grown on Transwell filters were apically infected with *G. lamblia*, and apical and basolateral secretion of CCL20 was measured by ELISA. The bars represent the means \pm standard deviations of three independent experiments. C) Secretion of CCL20 is activated by *G. lamblia* secreted factor(s). Differentiated Caco-2 cells were separated from *G. lamblia* by Transwell filters, and secreted CCL20 was measured by ELISA. The bars represent the means \pm standard deviations of three independent experiments.

bowel syndrome (IBS) (9), and since a mucosal immune activation can be seen in some IBS patients (4), it is possible that the induced chemokines can affect the outcome of IBS. Little inflammation is seen during a *Giardia* infection (23), and it is possible that *Giardia* can actively down-regulate the inflammatory response. Studies of biopsy material from giardiasis patients and experimental infections in knock-out mice will be required to further determine the role of these chemokines during giardiasis.

Induced stress and reduced proliferation in host cells during *G. lamblia* interaction. The largest number of genes with

changed expression are involved in regulation of stress and/or hypoxia and cellular proliferation. Stress-induced genes like c-Fos and c-Jun, immediate-early response 3 (IER3), hypoxia-inducible factor 2 (HIG2), enolase 1 and 2, and aldolase A and C are increased by time (supplemental material S3). Many genes involved in cellular proliferation (e.g., G0S2, PCNA, ORC5L, MCM2, and MCM3) are reduced after 6 and 18 h of incubation (supplemental material S3).

The detected stress and proliferation responses could be a consequence of reactive oxygen species (ROS) produced by the Caco-2 cells in response to the parasite. ROS can be produced by intestinal epithelial cells, and it has been proposed to be important for protection against intestinal infections (14). An interesting observation was that *Giardia* is more aerotolerant in the presence of tissue culture cells (data not shown). This has been observed earlier when anaerobic bacteria interact with human epithelial cells (15). It was proposed that the bacteria induce stress-responsive pathways as a response to ROS produced by the epithelial cells and that these pathways protect the parasite against oxygen stress (15). It remains to be seen if this is also the case during *Giardia*-host cell interactions.

The stress can also be a consequence of arginine depletion. *G. lamblia* uses arginine as its main energy source, and arginine starvation is induced already after 4 h of coincubation with Caco-2 cells (12). This is also a way for the parasite to avoid harmful nitric oxide (NO) production (12). Inducible nitric oxide (iNOS) is expressed in the Caco-2 cells during interaction with *Giardia*, and so is NADPH oxidase 1 (NOX1), a known producer of ROS (supplemental material S1 and S3). Real-time RT-PCR confirmed that they are expressed throughout the interaction, even if they are down-regulated at 18 h (iNOS, threefold; NOX1, fourfold; supplemental material S4). In human duodenal enterocytes, reduced arginine availability induces superoxide and peroxynitrite production by nitric oxide synthase (21, 22). These reactive substances could induce stress in the intestinal cells, in the end resulting in apoptosis. Induction of apoptosis and an increased intestinal permeability has been suggested to be a disease mechanism in giardiasis (7, 27). Interestingly, long-term arginine starvation of enterocytes induces apoptosis, which subsequently results in an increased intestinal permeability (25). Therefore, the symptoms achieved in giardiasis may be a consequence of local stress in the intestinal epithelial cells induced by reactive oxygen species.

In summary, our data show that coincubation of *G. lamblia* with cultured intestinal cells results in large changes in tissue cell gene expression. This will be the basis for future directed studies of both in vitro and in vivo *G. lamblia*-epithelial cell interactions, working toward deeper understanding of the mechanisms of this debilitating and costly disease.

ACKNOWLEDGMENTS

We thank David Brodin and David Reiner for helpful discussions.

This project was supported by a grant from the Swedish Medical Research Council (VR-M) to S.G.S. and the Wallenberg Consortium North (WCN).

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