J Physiol 593.24 (2015) pp 5269–5282

The Journal of Physiology

Interleukin-13 affects the epithelial sodium channel in the intestine by coordinated modulation of STAT6 and p38 MAPK activity

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Key points

- Interleukin-13 (IL-13) causes intestinal epithelial barrier dysfunction, and is implicated in the pathogenesis of Th2-driven intestinal inflammation (e.g. ulcerative colitis). However, it is unclear whether the epithelial sodium channel (ENaC) – the main limiting factor for sodium absorption in the distal colon – is also influenced by IL-13 and if so, by what mechanism(s).
- We demonstrate in an intestinal cell model as well as in mouse distal colon that IL-13 causes reduced ENaC activity.
- We show that IL-13 impairs ENaC-dependent sodium transport by activating the JAK1/2–STAT6 signalling pathway.
- These results improve our understanding of the mechanisms through which IL-13 functions as a key effector cytokine in ulcerative colitis, thereby contributing to the distinct pathology of this disease.

Abstract Interleukin-13 (IL-13) has been strongly implicated in the pathogenesis of ulcerative colitis, possibly by disrupting epithelial integrity. In the distal colon, the epithelial sodium channel (ENaC) is an important factor in the regulation of sodium absorption, and therefore plays a critical role in minimizing intestinal sodium and water losses. In the present study, we investigated whether IL-13 also acts as a potent modulator of epithelial sodium transport via ENaC, and the signalling components involved. The effect of IL-13 on ENaC was examined in HT-29/B6-GR/MR human colon cells, as well as in mouse distal colon, by measuring amiloride-sensitive short-circuit current (I_{SC}) in Ussing chambers. The expression levels of ENaC subunits and the cellular components that contribute to ENaC activity were analysed by qRT-PCR and promoter gene assay. We show that IL-13, in both the cell model and in native intestinal tissue, impaired epithelial sodium absorption via ENaC (J_{Na}) as a result of decreased transcription levels of β- and γ-ENaC subunits and SGK1, a post-translational regulator of ENaC activity, due to impaired promoter activity. The reduction in J_{Na} was prevented by inhibition of JAK1/2–STAT6 signalling. This inhibition also affected the IL-13-induced decrease in p38 MAPK phosphorylation. The contribution of STAT6 to IL-13-mediated ENaC inactivation was confirmed in a STAT6$^{-/-}$ mouse model. In conclusion, these results indicate that IL-13, the levels of which are elevated in ulcerative colitis, contributes to impaired ENaC activity via modulation of the STAT6/p38 MAPK pathways.

Please note that Figures 1, 3, 4, 5 and 6 that appear in this final copy-edited version have been updated since the paper was published online as an Accepted Article. The new figures have been supplied by the authors and have been approved by the Editor. The conclusions of the study remain the same.

DOI: 10.1113/JP271156

(Received 23 June 2015; accepted after revision 28 August 2015; first published online 14 September 2015)
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Abbreviations DBA, dexamethasone plus butyrate and aldosterone; ENaC, epithelial sodium channel; GR, glucocorticoid receptor; GRE, glucocorticoid response element; HRE, hormone response element; IL-13, interleukin-13; I_{SC}, amiloride sensitive short-circuit current; J_{Na}, epithelial sodium absorption via the ENaC; MAPK, mitogen-activated protein kinase; MR, mineralocorticoid receptor; MRE, mineralocorticoid response element; PI3K, phosphatidylinositol 3-kinase; SGK1, serum and glucocorticoid-induced kinase 1; TER, transepithelial resistance; UC, ulcerative colitis; WT, wildtype.

Introduction

The extent of water absorption by healthy human colon is directly related to the absorption of solutes, especially sodium ions (Na^+). Different Na^+ transport systems exist along the length of the large intestine. In the proximal colon, Na^+ is transported through electroneutral NaCl absorption, achieved by the sodium/hydrogen exchanger 3 (NHE3) acting in conjunction with chloride/bicarbonate antiporters (DRA or AE1). In distal colon, Na^+ is absorbed predominantly via the epithelial sodium channel (ENaC).

At a molecular level, ENaC is usually assumed to be a heterotrimer of one α-subunit, one β-subunit and one γ-subunit (Jasti *et al.* 2007; Firsov *et al.* 1998). In contrast to other steroid-responsive organs, such as lung and distal nephron, in the distal colon only the β- and γ-subunits are regulated, while the α-subunit is constitutively expressed (Renard *et al.* 1995; Epple *et al.* 2000). In addition, ENaC-dependent Na^+ transport is regulated by increased recruitment as well as prolonged insertion of active channels into the apical enterocyte membrane (Greig *et al.* 2004).

It has been shown that the apical ENaC β- and γ-subunits are down-regulated in ulcerative colitis (UC), and therefore contribute to the pathogenesis of diarrhoea in this disease (Amasheh *et al.* 2004; Sullivan *et al.* 2009). Furthermore, Amasheh and colleagues showed that ENaC dysregulation could be attributed to elevated proinflammatory cytokines, such as TNFα and interleukin-1β. In addition, IL-13 has been identified as a key inflammatory cytokine in the Th2 response in UC (Fuss *et al.* 2004). IL-13 leads to epithelial barrier disturbance, caused by increased epithelial apoptosis and also by an increased expression of the channel-forming tight junction protein claudin-2 (Heller *et al.* 2005). It has also been proposed that IL-13 is linked to the pathogenesis of Th2-cytokine mediated inflammation, since IL-13 neutralization prevented the inflammatory response in the oxazolone-colitis model (Heller *et al.* 2002). IL-13 acts through a heterodimeric cell-surface receptor composed of the IL-4Rα chain and the IL-13Rα1 chain. Its binding activates Janus-kinases (JAK) and leads to the phosphorylation and activation of signal transducers and activators of transcription (e.g. STAT3 and STAT6), which then dimerize and translocate to the nucleus, where they interact with other transcription factors to regulate gene transcription (Takeda *et al.* 1996; Hershey, 2003). Recent findings suggest that IL-13 binds to another receptor, the IL-13Rα2 chain (thought to act only as a decoy-receptor), which results in the activation of signalling cascades, such as AP1 or MAPK (Fichtner-Feigl *et al.* 2006; Mandal & Levine, 2010), both of which have been shown to regulate ENaC (Shen & Cotton, 2003; Zeissig *et al.* 2008). Previous observations suggest an important role for the STAT6 pathway activated by IL-13 in patients with UC, such as IL-13-induced epithelial apoptosis and claudin-2 expression *in vitro* (Rosen *et al.* 2011; Rosen *et al.* 2013).

Previous studies on the role of IL-13 in UC have only considered barrier destruction, and the influence of this cytokine on intestinal transport processes (for example, electrogenic Na^+ absorption), remains unclear. In allergic airway diseases, IL-13 acts as a potent modulator of ENaC activity in the lung through a STAT6-dependent mechanism (Anagnostopoulou *et al.* 2010). However, many differences exist between ENaC regulation in the lung and in the distal colon, and between the pathogenesis of allergic airway diseases and UC. Therefore, the aim of the current study was to evaluate whether ENaC function in the colon is regulated by IL-13, and the signalling pathways involved. In HT-29/B6-GR/MR cell monolayers (a novel, highly differentiated colon cell model) stably transfected with the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) (Bergann *et al.* 2011), we demonstrated that intestinal sodium absorption via ENaC was significantly affected by IL-13, a change which reflected defective up-regulation of ENaC β- and γ-subunits. Using inhibitors to differentiate between MAPK and JAK/STAT6 pathways, and STAT6-deficient ($STAT6^{-/-}$) mice, we found that IL-13-dependent dysregulation of ENaC relied on both MAPK (p38) and STAT6 signalling.

Methods

Ethical approval and animals

Ethical approval for animal experiments was obtained from the local ethics commitee of the LAGeSo (Landesamt für Gesundheit und Soziales, Berlin,

Germany; approval number: G 0208/12). BALB/c wild-type (Forschungseinrichtungen für experimentelle Medizin, Charité – Universitätsmedizin Berlin, Germany) and STAT6$^{-/-}$ mice on BALB/c background (The Jackson Laboratory, Bar Harbor, ME, USA) were maintained according to institutional standards. For cytokine stimulation experiments, 12- to 20-week-old female mice were administered an intraperitoneal (I.P.) injection of 3 μg recombinant murine IL-13 (BioLegend GmbH) dissolved in 300 μl phosphate buffered saline (PBS) or an equal volume of vehicle alone. Seventy-two hours later, further injections were administered once daily for three consecutive days. Twenty-four hours after the last challenge, mice were killed by cervical dislocation. Distal colon was removed for direct measurements in Ussing chamber experiments.

Cell culture and reagents

HT-29/B6-GR/MR cells, a subclone of the human colorectal cancer cell line HT-29 (Bergann *et al.* 2011), were cultured at 37°C in humidified 95% air/5% CO_2 in 25 cm^2 culture flasks in RPMI1640 medium supplemented with 10% fetal calf serum (FCS, Sigma-Aldrich Chemie GmbH, Munich, Germany), 1% penicillin–streptomycin (Sigma-Aldrich), 500 IU ml^{-1} G418 (Biochrom GmbH, Berlin, Germany) and 200 μg ml^{-1} hygromycin B (Life Technologies GmbH, Darmstadt, Germany). For most of the experiments cells were seeded on Millicell PCF filters (3 μm; Millipore, Schwalbach, Germany) and grown for 7 days. Experiments were performed when cell monolayers had reached transepithelial resistance (TER) values of 1500–2000 Ω cm^2. Prior to stimulating the cells with DBA (a combination of dexamethasone (D, 50 nM; Sigma-Aldrich), sodium butyrate (B, 2 mM; Merck-Schuchardt, Hohenbrunn, Germany) and aldosterone (A, 3 nM; Sigma-Aldrich)), they were incubated in medium containing 10% hormone-free FCS (Sigma-Aldrich) for 24 h. Human recombinant IL-13 (BioLegend GmbH, Fell, Germany) was added to the culture medium at 100 ng ml^{-1} 2 h after stimulating with DBA. In some experiments, cells were pre-treated with inhibitors for 2 h prior to exposure to IL-13. Specific MAPK inhibitors U0126 (which inhibits the p42/44 extracellular signal-regulated kinase, upstream kinase MEK 1/2) and SP600125 (which inibits JNK MAPK) (both from Cell Signaling Technology, Frankfurt am Main, Germany), and SB202190 (which inhibits p38 MAPK) (Calbiochem, Darmstadt, Germany), were used at the same concentration (10 μM). Baricitinib (500 nM; Selleckchem, Houston, TX, USA) was used to inhibit JAK 1 and 2 (JAK1/2). STAT3 inhibitor V (Stattic, 10 μM, Santa Cruz, Heidelberg, Germany) and AS1517499 (10 μM; Axon Medchem, Groningen, Netherlands) were used to inhibit STAT3 and STAT6, respectively, and Ly294002 (10 μM; Sigma-Aldrich) to inhibit the phosphatidylinositol 3-kinase (PI3K) pathway. The IL-13 type-I receptor (IL-4Rα/IL-13Rα1) was blocked with an anti-IL-4R antibody (100 ng ml^{-1}; R&D Systems, Wiesbaden, Germany).

Electrophysiological measurements

Human epithelial cell culture. After 24 to 96 h of stimulation, confluent monolayers of HT-29/B6-GR/MR cells grown on filters were mounted into Ussing-type chambers (epithelial area 0.6 cm^2). The bathing solution consisted of (mM): Na^+ 140.0; Cl^- 123.8; K^+ 5.4; Ca^{2+} 1.2; Mg^{2+} 1.2; HPO_4^{2-} 2.4; $H_2PO_4^-$ 0.6 and HCO_3^- 21.0. The solution was gassed with a 95% O_2/5% CO_2 mixture by bubble lift. Temperature was kept at 37°C, pH 7.4. Transepithelial resistance (TER, Ω cm^2) and short-circuit current (I_{SC}; μA cm^{-2}) were recorded using voltage clamp devices (CVC6; Fiebig, Berlin, Germany). After an equilibration period of 20 min, the ENaC-dependent Na^+ transport was measured as a drop in I_{SC} after adding the ENaC blocker amiloride (100 μM; Sigma-Aldrich) to the apical side of the cell monolayer. The concentration of amiloride yielding 50% ENaC inhibition (IC_{50}) is ~100 nM (Kellenberger & Schild, 2002). To ensure complete inhibition of ENaC in the colonic epithelium, which is covered by a mucus layer, an amiloride concentration of 100 μM was employed. This concentration is 10-fold higher than concentrations usually used to completely block the ENaC in epithelial cell models, or in the kidney (10 μM), but still specific for Na^+ transport via the ENaC, as NHE3, the other transport system for Na^+ in the apical cell membrane of colonocytes, is only affected by amiloride concentrations at 1 mM.

Mouse colon. For determination of the electrogenic Na^+ absorption in the colon of BALB/c and STAT6$^{-/-}$ mice, epithelia were mounted into miniaturized Ussing chambers with the exposed area of 0.049 cm^2. The bathing solution had the same ion composition described above. Additionally, 10.0 mM D(+)-glucose, 10.0 mM D(+)-mannose, 2.5 mM glutamine and the antibiotics piperacillin (50 mg l^{-1}) and imipenem (4 mg l^{-1}) were added. After a 30 min equilibration period with heat-inactivated hormone-free FCS (10%), which prevented adhesion of cytokines to glass surfaces of the Ussing set-up, IL-13 (100 ng ml^{-1}) was added to the serosal side of the epithelia. After an additional 30 min, the epithelia were stimulated by adding DBA to both sides of the epithelium. After 8 h, amiloride (100 μM) was added to the mucosal compartment to quantify ENaC-dependent electrogenic sodium absorption (J_{Na}).

RNA extraction and real-time RT-PCR. In accordance with the manufacturer's protocol, total RNA was isolated

by using PeqGOLD RNApure (PEQLAB, Biotechnologie GmbH, Germany). Two micrograms of extracted RNA were then reverse-transcribed into cDNA by using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Mannheim, Germany), following the manufacturer's instructions. mRNA expression of the β- and γ-ENaC subunits and SGK-1 under different conditions was evaluated by real-time PCR, using a 7500 Fast Real-time PCR System and TaqMan Gene Expression assays No. HS00165722_ml, HS00168918_ml and HS00178612_ml (Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (unlabelled primer, VIC/TAMARA) served as a reference gene. Relative mRNA expression for each transcript of interest was quantified using the comparative C_t ($\Delta\Delta C_t$) method (Livak & Schmittgen, 2001).

Transfection and gene reporter assay

HT-29/B6-GR/MR cells were seeded with a density of 5×10^4 cells/12-well under hormone-free conditions. After 24 h, transient transfections were performed with Lipofectamine plus (Invitrogen, Karlsruhe, Germany). The reporter gene construct pMMTV-Luc, containing tandem hormone response element (HRE) sequences followed by a luciferase (luc) gene, served for the detection of HRE-driven luciferase expression. The pghENaC-B construct (−459 to +35) was a gift of Dr Christie P. Thomas (University of Iowa, Iowa City, IA, USA), and was used to study the promoter activity of γ-ENaC. SGK1-driven luciferase expression was detected using a phSGK1-3kb-up-Luc construct containing the SGK1 promoter sequence followed by firefly luciferase gene (a gift of Dr Tim Zierra, Bayer Schering Pharma AG, Berlin). As co-reporter, a pGL4.70 construct (hRluc; Promega, Mannheim, Germany) containing a *Renilla* luciferase gene was applied. The expression level of *Renilla* luciferase remained unaffected under our experimental conditions, and served to normalize the transfection efficiency.

A total of 500 ng reporter plasmid and 50 ng of co-reporter plasmid per well were used for transfection. Four hours after transfection, cells were stimulated with DBA, and 2 h later with rhIL-13. Measurement of both firefly and *Renilla* luciferase activity was performed with the Dual-Luciferase Reporter Assay System (Promega) and a centro LB 960 microplate-luminometer (Berthold, Bad Wildbach, Germany) 72 h after transfection.

Western blot analysis

Two hours after stimulation of filter-grown HT-29/B6-GR/MR cells with DBA in completely hormone-free medium, rhIL-13 (100 ng ml^{-1}) was added to the basal side of the cells. They were then incubated for 96 h. The medium was then replaced by medium lacking FCS and IL-13. Cells were incubated again for 3 h prior to a new incubation with IL-13. Depending on the phosphorylated protein, after 5, 15, 30 or 60 min of IL-13 incubation whole cell protein was prepared. In brief, cells were washed with ice-cold PBS and scraped off with lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM Triton X-100, 1 mM EDTA, 1 mM PMSF, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM EGTA, complete protease inhibitor cocktail (Roche, Mannheim, Germany)) and pushed through a 1 ml hypodermic syringe with a 0.45 × 10 mm needle. After an incubation step of 10 min on ice, the lysates were centrifuged (15,000 *g*, 15 min, 4°C) and the supernatants used as whole cell extracts. Proteins were resolved by SDS-Page on a 12% gel under reducing conditions and electrotransferred onto PVDF-membranes. After blocking for 1 h in blocking solution (5% BSA/PBS-T), membranes were incubated with primary antibodies overnight at 4°C. Antibodies for detection of anti-phospho-p42/44 MAPK (anti-pERK; Thr202/Tyr204), anti-phospho-SAP/JNK (anti-pJNK; Thr183/Tyr185), anti-phospho-p38 (anti-p-p38; Thr334), anti-p42/44 MAPK (anti-ERK; Thr202/Tyr204), anti-SAPK/JNK (anti-JNK), anti-p38 (Thr334), and phospho-specific and total STAT6 and STAT3 were purchased from Cell Signaling Technology. Anti-β-actin was purchased from Sigma-Aldrich. Following an incubation period for 1 h at room temperature with the peroxidase (POD)-conjugated secondary antibody (Lumi-Light^{PLUS} Western blotting kit; Roche), chemiluminescence signals were detected using an Fusion FX7 imaging system (Vilber Lourmat Deutschland GmbH, Eberhardzell, Germany), and were analysed with the AIDA program package (Raytest, Berlin, Germany).

Statistical analysis

Data are presented as arithmetic means ± standard error of the mean (SEM) and were evaluated using student's t-test for independent samples. For comparison of more than two groups, analysis of variance was performed (1-way ANOVA) for non-parametric data, or was adjusted with the Bonferroni correction for parametric values. *P*-values < 0.05 were considered to be statistically significant.

Results

IL-13 impairs ENaC-dependent active electrogenic Na^+ transport (J_{Na}) in colonic HT-29/B6-GR/MR cells and native mouse colon

To explore the IL-13 effect on J_{Na}, HT-29/B6-GR/MR cells were treated with DBA (D: dexamethasone, 50 nM; B: sodium butyrate, 2 mM; A: aldosterone, 3 nM) in order

to stimulate J_{Na}. After 96 h with and without IL-13, Ussing measurements were performed. IL-13 (100 ng ml^{-1}) reduced the DBA-induced J_{Na} to 49 ± 9% (Fig. 1*A*). Interestingly, this pronounced effect of IL-13 was observed only after 96 h (Fig. 1*B*), whereas the transepithelial resistance (TER) was already reduced after 48 h (1527 ± 116 Ω cm^2 for DBA *versus* 1094 ± 132 Ω cm^2 for DBA+IL-13; $P < 0.05$). Furthermore, the IL-13 influence on J_{Na} was dose dependent, with significant effects starting at 50 ng ml^{-1} (Fig. 1*C*). Since malignant transformation may alter electrogenic Na^+ transport in colonic cell lines, we also studied the effect of IL-13 in native large intestine from BALB/c mice. Wild-type (WT) mice were I.P. pre-treated with either IL-13 or vehicle for 1 week. *Ex vivo* distal colon specimens were then stimulated with DBA in the Ussing chamber (Fig. 1*D* and *E*). Similar to the effects in HT-29/B6-GR/MR cells, J_{Na} was also decreased by IL-13 in distal mouse colon (1.8 ± 0.4 μmol h^{-1} cm^{-2} for IL-13 *versus* 4.0 ± 1.0 μmol h^{-1} cm^{-2} for vehicle; $P < 0.01$).

IL-13 effects on ENaC activity are apoptosis independent but due to reduced transcription of β- and γ-ENaC subunits and SGK1

In order to exclude the possibility that IL-13-suppression of J_{Na} simply resulted from cell damage due to induction of epithelial apoptosis, caspase-3 cleavage was quantified. However, IL-13 did not activate apoptosis in HT-29/B6-GR/MR cells, at least at the dose of 100 ng ml^{-1} and an incubation time of 96 h (Fig. 2*A*). To analyse whether the observed effects of IL-13 could be attributed to changes in ENaC mRNA expression, the expression level of ENaC subunit mRNAs was examined by real time-PCR. The increase in β- and γ-ENaC mRNAs was smaller in HT-29/B6-GR/MR cells incubated with DBA+IL-13 (321 ± 70-fold of control for β-ENaC, and 601 ± 161-fold of control for γ-ENaC) than in cells incubated solely with DBA (610 ± 98-fold for β-ENaC and 1116 ± 155-fold for γ-ENaC) (Fig. 2*B*). By contrast, the DBA-induced increase in α-ENaC subunit expression was marginal

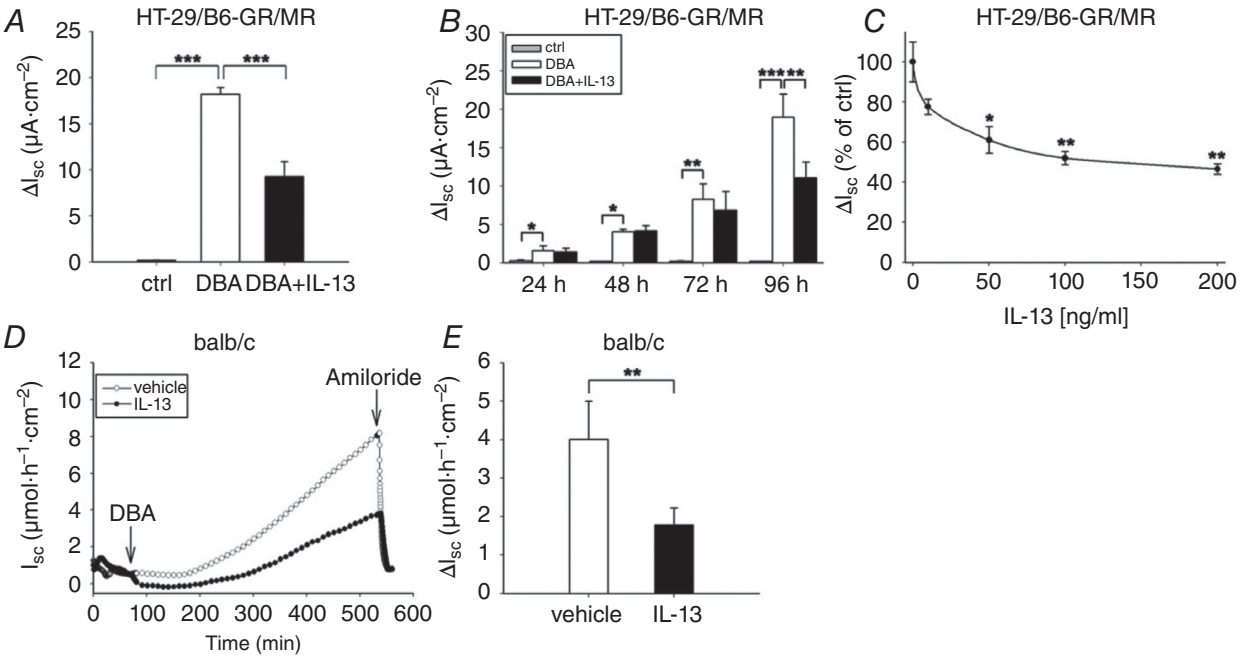

Figure 1. IL-13 impairs ENaC-dependent Na^+ absorption in HT-29/B6-GR/MR cells as well as in native intestinal tissue
A–C, cells were non-treated (ctrl) or triple-incubated (DBA, dexamethasone (50 nM), butyrate (2 mM), aldosterone (3 nM)) and treated with or without IL-13. *A*, ENaC-dependent Na^+ absorption after 96 h of incubation (J_{Na}) was measured as drop in I_{SC} after addition of amiloride (100 μM). *B*, time-dependent progress of J_{Na}. *C*, IL-13 dose dependency of J_{Na}. Data in *A–C* represent means ± SEM; n = 4–10, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. *D*, time course of a representative experiment of DBA-induced active electrogenic Na^+ transport in the distal colon of WT mice that were pretreated with PBS (ctrl) or IL-13 as described in Methods. Tissues were stimulated with a triple combination of DBA for 8 h and then ENaC-dependent Na^+ absorption (J_{Na}) was determined as drop in amiloride-sensitive I_{SC}. *E*, J_{Na} of WT mice treated as described in *D* given as means ± SEM of 8 mice per group; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

and not affected by the additional incubation of IL-13 (2.9 ± 0.4-fold of control for DBA *vs.* 3.3 ± 0.6-fold for DBA with IL-13, $P = 0.5$).

An important intracellular regulator of J_{Na} is the serum and glucocorticoid-regulated kinase-1 (SGK1), which triggers the apical localization of ENaC and is regulated by glucocorticoids (GC) and mineralocorticoids (MC) (Chen *et al.* 1999; Itani *et al.* 2002). Determination of the SGK1 expression level demonstrated the DBA-induced mRNA expression to be attenuated by IL-13 (47 ± 6-fold of control for DBA *versus* 26 ± 5-fold of control for DBA+IL-13; $P < 0.05$; Fig. 2*C*). To further investigate whether the effect of IL-13 on β- and γ-ENaC subunits or SGK1 gene expression was due to altered transactivation by the GR or MR, HT-29/B6-GR/MR cells were transiently transfected with the plasmid pMMTV-Luc, containing consensus HRE sequences in the promoter region of a luciferase gene. However, even after an incubation period of 72 h, IL-13 did not modify the DBA-dependent upregulation of HRE-driven luciferase expression (Fig. 2*D*). To study the effect of IL-13 on γ-ENaC promoter, HT-29/B6-GR/MR cells were transiently transfected with the plasmid pghENaC-B (phγENaC-Luc), linking an appropriate γ-ENaC promoter fragment to a luciferase reporter gene. Spontaneous transcription activity of γ-ENaC was significantly reduced in the presence of IL-13 (68 ± 3%; $P < 0.001$ compared to control; Fig. 2*E*), indicating a regulatory effect of IL-13 on γ-ENaC transactivation. To investigate the possibility of associated changes in SGK1 promoter activity, HT-29/B6-GR/MR cells were transfected with the phSGK1-3kb-up-Luc plasmid (phSGK1-Luc) and incubated with DBA with and without IL-13. Cells exposed to IL-13 and DBA showed decreased luciferase expression levels (377 ± 29-fold for DBA+IL-13 *versus* 521 ± 19-fold for DBA; $P < 0.001$). This indicates that IL-13 led to decreases in both γ-ENaC subunit and SGK1 mRNA expression, which could be attributed to IL-13-mediated changes of promoter activity.

IL-13 influences all MAPKs but only p38 signalling is involved in J_{Na} regulation

MAPK pathways can be activated by IL-13 (David *et al.* 2001; Mandal & Levine, 2010) and MAPKs are involved in transcriptional, translational and post-translational ENaC regulation (Rogatsky *et al.* 1998; Shen & Cotton, 2003; Yang *et al.* 2006; Zeissig *et al.* 2008). To elucidate whether IL-13 affected MAPK signalling in our HT-29/B6-GR/MR

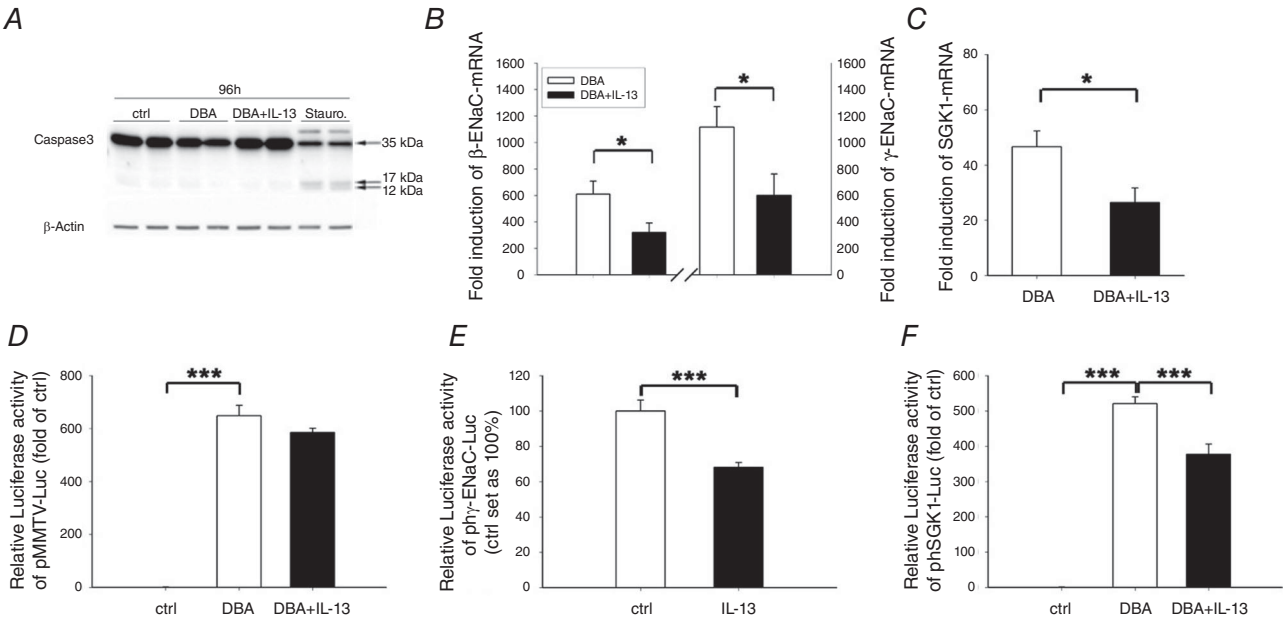

Figure 2. ENaC-mediated Na^+ absorption is inhibited by IL-13 which was apoptosis independent but due to decreased ENaC subunit and SGK1 transcription
A, apoptosis control. HT-29/B6-GR/MR cells were incubated without (ctrl) or with DBA and with or without IL-13 (100 ng ml^{-1}), and caspase-3 activation/cleavage was studied by Western blot analyses. Staurosporine served as positive control and human β-actin as loading control. $n = 4$. *B* and *C*, cells were incubated with or without DBA and with or without IL-13, and mRNA expression of β- and γ-ENaC subunits (*B*) and SGK1 (*C*) was measured after an incubation period of 72–96 h. *D–F*, for gene reporter assay, cells were transfected with either pMMTV-Luc (*D*), pgh-ENaC-B (*E*), or phSGK1-3kb-up-Luc (*F*) and incubated for 72 h with or without DBA in the presence or absence of IL-13 (100 ng ml^{-1}). Data given are means ± SEM of *x*-fold induction over controls; $n = 4$–5, $^*P < 0.05$, $^{***}P < 0.001$.

cell model, MAPK phosphorylation/activation was examined with phospho-specific antibodies. All three MAPKs were affected by IL-13 (Fig. 3*A* and *B*). ERK1/2 and JNK phosphorylation was augmented by IL-13 (ERK1/2: 62 ± 2% of control for DBA *versus* 98 ± 2% of control for DBA+IL-13; JNK: 96 ± 5% of control for DBA *versus* 229 ± 14% of control for DBA+IL-13, Fig. 3*B*). By contrast, p38 phosphorylation, which was strongly increased after stimulation with DBA, was inhibited by IL-13 (598 ± 78% of control for DBA *versus* 262 ± 44% of control for DBA+IL-13; $P < 0.01$). Having analysed MAPK phosphorylation, we tested whether IL-13 modulation of MAPKs was also reflected in J_{Na} by performing Ussing experiments with specific MAPK inhibitors. Neither the ERK1/2 inhibitor (U0126) nor the JNK inhibitor (SP600125) prevented the IL-13-mediated suppression in J_{Na}, and both inhibitors also had no significant effect on J_{Na} in the absence of IL-13 (Fig. 3*C*). However, pre-incubation with the p38 inhibitor SB202190 markedly decreased the DBA-induced J_{Na} (Fig. 3*D*). Under these conditions, the IL-13 effect on ENaC activity was completely abrogated (20 ± 1% of DBA for DBA with inhibitor *versus* 22 ± 4% of DBA for DBA with inhibitor plus IL-13. It should be emphasized that despite the substantial decrease in DBA-stimulated J_{Na} produced by p38 inhibition, J_{Na} remained significantly increased compared with control cells.

The JAK/STAT(6) pathway mediates the IL-13-mediated inhibition of J_{Na}

To elucidate the mechanisms underlying the IL-13-mediated decrease in J_{Na}, we investigated the STAT6-pathway, which is one of the major effector

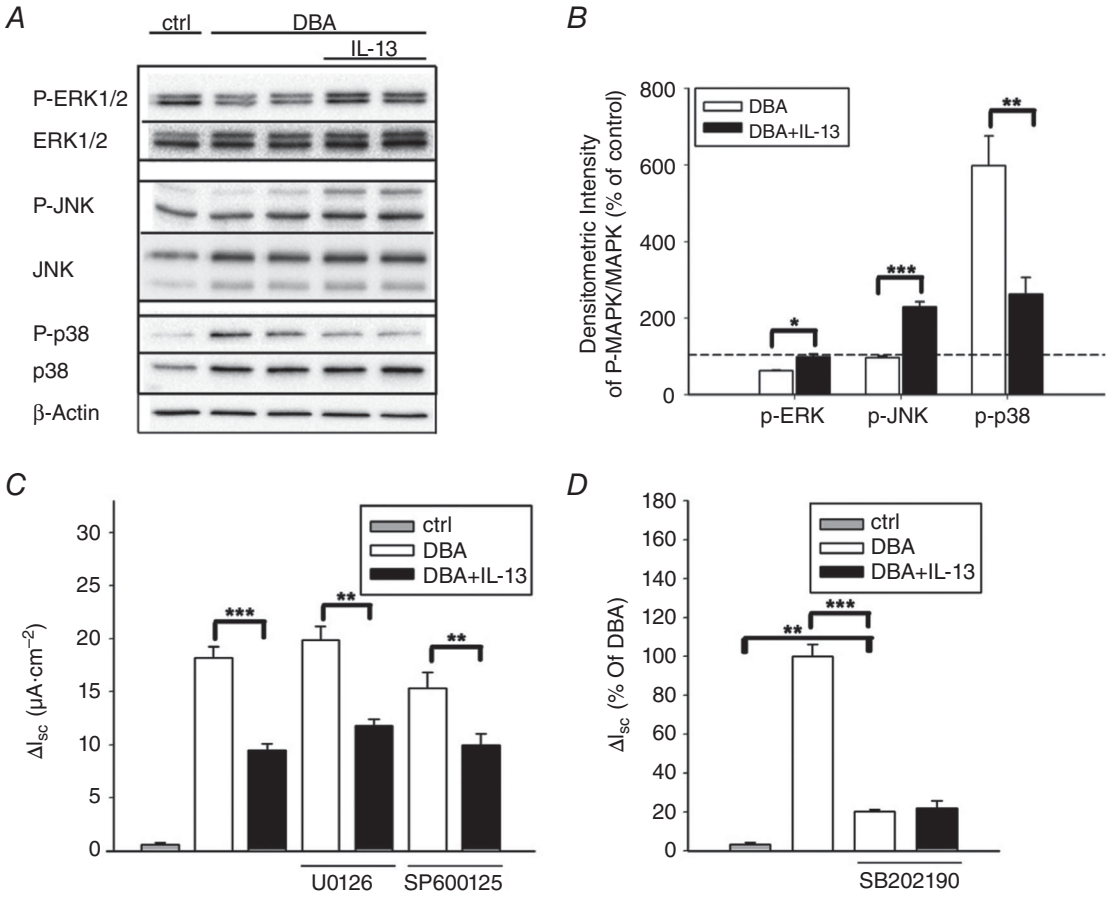

Figure 3. ENaC-mediated Na^+ absorption is suppressed by IL-13 via inhibition of p38 activity
A, representative Western blot analysis of both phosphorylated and total ERK1/2, JNK, or p38 proteins after IL-13 exposition. HT-29/B6-GR/MR cells were treated with or without DBA and with or without IL-13 for 96 h, then rested in media lacking FCS and IL-13 for 3 h and stimulated again with IL-13 for 5 min (ERK), 15 min (JNK) or 60 min (p38). Then, total cellular protein was extracted and analysed. *B*, densitometric measurements were obtained from 3–4 blot membranes representing independent experiments. Data given as means ± SEM; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. *C*, determination of ENaC-dependent Na^+ absorption in the presence or absence of ERK1/2 (U0126) or JNK (SP600125) inhibitors. *D*, determination of ENaC-dependent Na^+ transport with or without the p38 inhibitor SB202190. Data are given as means ± SEM; $n = 6–7$, $^{**}P < 0.01$, $^{***}P < 0.001$.

pathways of IL-13 (Hebenstreit *et al.* 2006). In addition, there are a few reports which describe an IL-13-dependent phosphorylation of STAT3 (Umeshita-Suyama *et al.* 2000). Thus, we performed Western blot studies with phospho-specific antibodies for both STAT pathways in HT-29/B6-GR/MR cells. STAT6 and STAT3 were both phosphorylated/activated by IL-13, whereas this phosphorylation was not induced by DBA (Fig. 4*A*).

Next we determined if the IL-13-dependent STAT activation was associated with changes in J_{Na}. As shown in Fig. 4*B*, Ussing experiments revealed that blocking STAT3 signalling by the STAT3 inhibitor V (Stattic) had no influence on the impaired J_{Na} after IL-13 exposure. By contrast, the STAT6-specific inhibitor AS1517499 (Chiba *et al.* 2009) led to complete disinhibition of the IL-13-mediated reduction in J_{Na} (16.6 ± 0.9 without IL-13 *versus* 14.5 ± 1.1 μA cm^{-2} with IL-13; $P = 0.322$). The JAK1/2 inhibitor baricitinib also abolished the IL-13-mediated decrease in J_{Na} (18.7 ± 1.2 without IL-13 *versus* 16.7 ± 0.8 μA cm^{-2} with IL-13; $P = 0.056$).

IL-13 signals through the IL-13- Rα1/IL-4Rα receptor in HT-29/B6-GR/MR

Because the IL-13-induced activation of the JAK1/2–STAT6 pathway is widely attributed to the heterodimeric type-I receptor (IL-13Rα1/IL-4Rα), we performed inhibitor experiments with an antibody against the IL-4Rα to confirm this in our cell line. This inhibitory antibody blocked the IL-13 effect on J_{Na} (DBA+IL-13 in the presence of the IL-4Rα-antibody: 86 ± 9% of DBA with antibody; DBA+IL-13 in the absence of the IL-4Rα-antibody: 45 ± 12% of solely DBA; $n = 6$ each; $P < 0.05$).

Since PI3K signalling has also been described by IL-13, we analysed the phosphorylation status of Akt (p-Akt Thr308), a downstream molecule of PI3K. In Western blots, this pathway was also activated by IL-13 in our cell model. However, when examining J_{Na} in the presence of the PI3K inhibitor Ly294002, no effect on IL-13-induced changes by PI3K inhibition was detected (18.8 ± 1.9 for DBA *versus* 13.9 ± 2.2 μA cm^{-2} for DBA+IL-13; $P < 0.05$).

STAT6 mediates the IL-13-induced inhibition of the p38 MAPK

In an earlier study, Jang and co-workers reported a relationship between p38 and STAT6 activation in a P388D1 macrophage cell line, as well as in BALB/c macrophages (Jang *et al.* 2009). To monitor the influence of STAT6 on the different MAP kinases, we analysed their phosphorylation status in cells stimulated with DBA with and without IL-13 in the presence or absence of baricitinib. Whereas baricitinib did not inhibit IL-13-induced ERK1/2 and JNK phosphorylation, the IL-13-mediated decrease in p38 phosphorylation was significantly attenuated by baricitinib (170 ± 13% of control for DBA *versus* 85 ± 3% of control for DBA+IL-13; $P < 0.01$; compared to 139 ± 9% of control for DBA+IL-13 with baricitinib; $P < 0.01$; Fig. 5*A* and *B*). The inhibition of JAK1/2 by baricitinib was accompanied by inhibition of STAT6 phosphorylation (Fig. 5*A*).

To exclude a STAT6-independent effect on p38 phosphorylation mediated via JAK1/2 inhibition, we monitored p38 phosphorylation under the influence of the STAT6-specific inhibitor AS1517499. Similar to JAK1/2 inhibition by baricitinib, AS1517499 led to disinhibition of p38 phosphorylation (329 ± 37% of control for DBA *versus* 137 ± 11% of control for DBA+IL-13; $P < 0.01$;

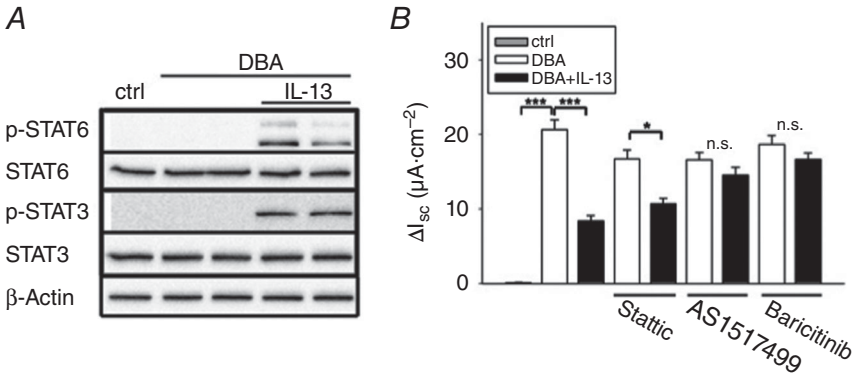

Figure 4. The JAK1/2/STAT signalling pathway is stimulated after IL-13 treatment in HT-29/B6-GR/MR cells and is responsible for the drop in ENaC-dependent Na^+ absorption
A, representative Western blot analyses of both phosphorylated and total STAT6 and STAT3. Cells were stimulated with DBA and with or without IL-13 for 96 h, then rested FCS and IL-13 free for 3 h and treated again with IL-13 for 30 min. Then, total cellular protein was extracted, fractionated by SDS-Page and immuno-probed for phospho-specific and total STAT6 and STAT3 expression. *B*, measurement of ENaC-dependent Na^+ absorption with or without IL-13 in presence of DBA and inhibitors of the JAK1/2/STAT signalling. Results are means ± SEM; $n = 5$–7, $^*P < 0.05$, $^{***}P < 0.001$.

compared to 220 ± 5% of control for DBA+IL-13 with AS1517499; $P < 0.01$; Fig. 5*C* and *D*).

To identify the order of STAT6 and p38 in the IL-13-induced signalling, we monitored STAT6 phosphorylation in response to IL-13 either with or without the p38 inhibitor SB202190. When present, STAT6 phosphorylation was still detectable at levels comparable to those without SB202190 (DBA+IL-13+SB202190: 88 ± 12% of DBA+IL-13 without SB202190; $n = 4$ each; $P = 0.48$). Thus, the IL-13-dependent inhibition of p38 phosphorylation was mediated via STAT6 activation.

IL-13 impairs J_{Na} in mouse distal colon via STAT6

For confirmation of the role of STAT6, we performed experiments in STAT6$^{-/-}$ BALB/c mice. Incubation with either vehicle or IL-13 led to a similar increase in J_{Na} in the knockout mouse (Fig. 6*A*). Direct comparison of the response to IL-13 in WT and STAT6$^{-/-}$ mice showed that the IL-13-mediated repression of J_{Na} was prevented in the STAT6 knockout mouse (WT: with IL-13 45 ± 11% of control; $P < 0.01$; STAT6$^{-/-}$: with IL-13 86 ± 12% of control; $P = 0.45$; Fig. 6*B*). These findings corroborate the results obtained in HT-29/B6-GR/MR cells, confirming that STAT6 signalling is essential for the IL-13 effect on J_{Na}.

Discussion

IL-13 is known to affect the intestinal epithelium, particularly by impairing epithelial barrier function (Heller *et al.* 2005; Prasad *et al.* 2005). It is produced by CD1-reactive NKT cells (Fuss, 2004), and is considered to be a key effector cytokine in Th2-driven colitis. Here, we show for the first time that IL-13 affects active electrogenic Na^+ transport (the most important Na^+ transport system in the human distal colon) as well as intestinal epithelial barrier function. Exposure to IL-13 reduced DBA-stimulated ENaC-dependent Na^+ absorption (J_{Na}) in our HT-29/B6-GR/MR cell model, and

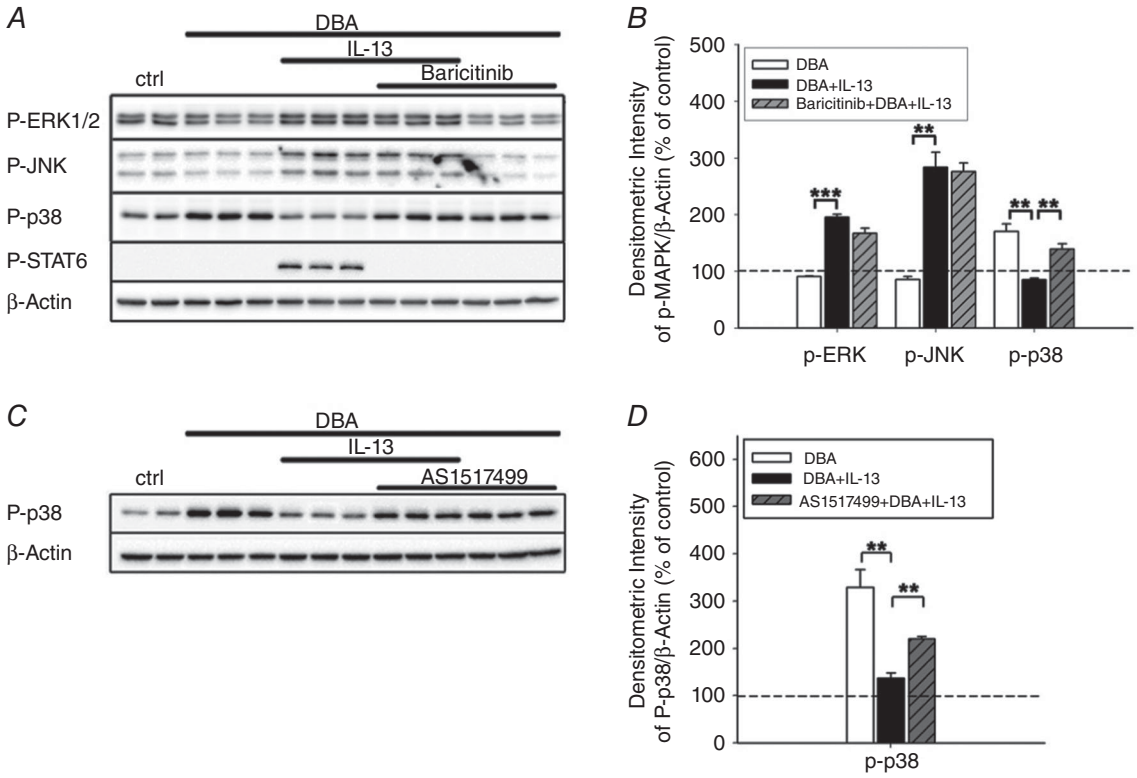

Figure 5. STAT6 mediates IL-13-dependent inhibition of p38 phosphorylation
HT-29/B6-GR/MR cells were stimulated with or without DBA and with or without IL-13 in the presence or absence of baricitinib or AS1517499 for 96 h, then rested FCS and IL-13 free for 3 h and treated again with IL-13 for 30 min. Then, total cellular protein was extracted, fractionated by SDS-Page, and immuno-probed for phospho-specific ERK1/2, JNK, p38, or STAT6 expression. Human β-actin served as loading control. *A* and *B*, representative Western blot analyses of the indicated proteins in the absence or presence of baricitinib (*A*) and the corresponding densitometric measurements (*B*). *C* and *D*, representative Western blot analyses of phospho-specific p38 MAPK in the absence or presence of AS1517499 (*C*) and the corresponding densitometric measurements (*D*). Results in *B* and *D* are means ± SEM; $n = 3$, $^{**}P < 0.01$, $^{***}P < 0.001$.

this was supported by *ex vivo* investigations in BALB/c mice. This effect of IL-13 was mediated by activation of STAT6, a second important finding of our present study, again supported by additional inhibitor and knockout experiments.

Under physiological conditions, intestinal electrogenic Na^+ transport occurs at only modest levels, although it plays a more important role during electrolyte and water loss in diarrhoeal states (Schultheis *et al.* 1998; Tatum *et al.* 2010). Under those conditions, ENaC is abundantly expressed and located in the apical membrane of enterocytes as the result of steroid action. Although different cellular processes, such as reduced ubiquitination, contribute to this up-regulation of ENaC activity, we and others have previously shown that regulation occurs to a significant extent via transcriptional induction of β- and γ-ENaC subunits, while the α-ENaC subunit is constitutively expressed (Renard *et al.* 1995; Epple *et al.* 2000).

To simulate conditions found in native intestine during induction of diarrhoea, and to characterize the maximum transport capacity of this transport system, we stimulated ENaC in our experimental models with a triple-combination of dexamethasone, butyrate and aldosterone (DBA). Butyrate has been shown to be involved in enterocyte differentiation and to act as a histone deacetylation inhibitor in the transcriptional up-regulation of ENaC (Cuff & Shirazi-Beechey, 2004). However, a detailed analysis revealed butyrate alone to be insufficient to activate ENaC-dependent Na^+ absorption in the absence of steroids (Zeissig *et al.* 2008; Bergann *et al.* 2009*a*). Although corticosteroids (for example, dexamethasone) alone can stimulate Na^+ absorption, their effect on electrogenic Na^+ absorption is synergistically enhanced by butyrate. In addition, earlier *in vivo* perfusion studies on adrenectomized rats demonstrated that corticosteroids represent a *conditio sine qua non* cofactor for maximum induction of ENaC activity by aldosterone (Fromm *et al.* 1990); indeed, basal levels of circulating glucocorticoid are always present *in vivo* (Fromm *et al.* 1990). Thus, the mineralocorticosteroid aldosterone, which is the most important regulator of electrogenic Na^+ absorption in the colon (especially during electrolyte and water losses associated with diarrhoea), requires co-stimulatory inputs from glucocorticoids and butyrate for maximum ENaC activation.

When glucocorticoids, butyrate and aldosterone were present at physiological concentrations, we found a substantial increase in J_{Na} in our HT-29/B6-GR/MR cell model that was severely impaired by IL-13, both changes reflecting regulation of β- and γ-ENaC subunit mRNA. Only ENaC molecules comprising all three subunits inserted into the apical colonocyte membrane allow Na^+ transport in an effective manner (Canessa *et al.* 1994; Renard *et al.* 1995). Our data obtained in the colon are consistent with findings of Anagnostopoulou and co-workers, who analysed ion transport processes in airway epithelium of mice after instillation of IL-13 (Anagnostopoulou *et al.* 2010), also a key effector molecule in the pathogenesis of allergic airway inflammation (Wills-Karp *et al.* 1998). Constitutively active in the lung, ENaC contributes to the generation of an osmotic Na^+ gradient that clears fluid from the alveolar space (Matthay *et al.* 2002), and administration of IL-13 also led to

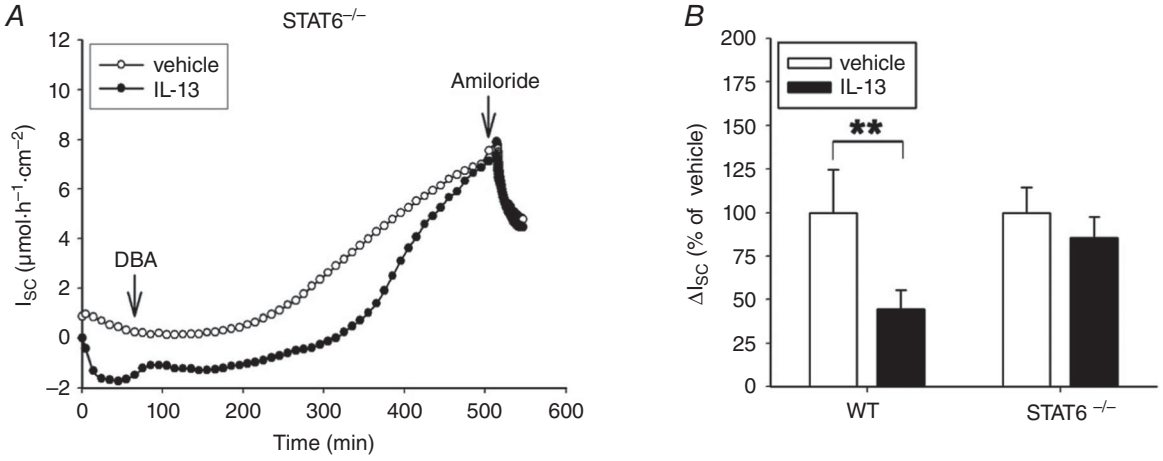

Figure 6. In mouse distal colon IL-13 affects the DBA-induced ENaC-dependent Na^+ absorption in a STAT6-dependent manner
A, time course of a representative experiment on electrogenic Na^+ transport (I_{SC}) in the distal colon of STAT6$^{-/-}$ mice, which were pre-treated with PBS (ctrl) or IL-13 as described in Methods. The tissue was stimulated with DBA for 8 h and then ENaC-dependent Na^+ absorption was determined as drop in I_{SC}. *B*, comparison of ENaC-dependent Na^+ absorption in WT and STAT6$^{-/-}$ mice treated as described in *A*. Data given as means ± SEM of 5–8 mice per group, $^{**}P < 0.01$.

impaired Na^+ transport. Resembling the results of our present study, this observation was also associated with a down-regulation of ENaC subunit mRNAs.

However, an increase in ENaC-mediated Na^+ absorption does not depend only on transcriptional up-regulation of ENaC subunits; important also are a number of regulatory proteins which contribute to rapid ENaC turnover in the apical cell membrane. In this regard, our finding of IL-13-mediated down-regulation of SGK1 mRNA indicated an effect of IL-13 on ENaC activity at the post-translational level. The stimulatory impact of SGK1 on ENaC is mediated via an inhibitory interaction with Nedd4-2 (neural precursor cell-expressed, developmentally down-regulated protein 4-2). As shown by others, Nedd4-2 facilitates the ubiquitination and subsequently the removal of ENaC from the apical cell membrane, leading to degradation of ENaC complexes (Debonneville *et al.* 2001).

Nevertheless, the relative contribution of IL-13-induced alterations of transcriptional and post-translational events to the measured decrease in ENaC-mediated Na^+ absorption has been unclear. On the one hand, as mentioned above, it is possible that decreased β- and γ-ENaC expression levels are responsible for ENaC dysfunction. On the other hand, it is also possible that down-regulation of SGK1 is the rate limiting factor. To distinguish between these possibilities, additional approaches would have been needed with pharmacological inhibitors of SGK1 or siRNAs at various levels of ENaC activation.

IL-13 actions via the heterodimeric type-I (IL-4Rα/IL-13Rα1) receptor, as identified here in our present study using neutralizing antibodies, have been shown for different target genes and in different tissues to involve phosphorylation of JAK1/2 and subsequently phosphorylation and activation of the transcription factors STAT3 and STAT6 (Umeshita-Suyama *et al.* 2000). Additionally, STAT-independent activation of the PI3K signalling cascade has been described (Nelms *et al.* 1999), but in our study did not appear to have a role for the inhibition of J_{Na}, as indicated by the absence of an effect of Ly294002. In the subsequent analysis, a role for STAT6, but not for STAT3, could be detected for ENaC activation in the colon.

In addition to these classical pathways, IL-13 is also known to signal via MAP kinases (David *et al.* 2001; Moynihan *et al.* 2008). Furthermore, MAP kinases have previously been shown to influence ENaC-dependent Na^+ absorption in intestinal cells (Zeissig *et al.* 2008; Bergann *et al.* 2009*b*; Kuntzsch *et al.* 2012). We found all three MAPKs were modified by IL-13, but inhibitor experiments indicated that only p38 MAPK was involved in the regulation of ENaC. Our finding that inhibition of JAK1/2 as well as STAT6 disinhibited this IL-13 effect, suggests that JAK/STAT6-mediated p38 MAPK suppression is involved, as shown in Fig. 7. Other investigators have also proposed a link between p38 MAPK and the JAK1/2–STAT6 pathway, as cytokine-mediated activation of p38 MAPK targeted STAT6 signals in a P388D1 macrophage cell line and in murine peritoneal macrophages (Jang *et al.* 2009; Jimenez-Garcia *et al.* 2015). Our present data lead us to conclude that in the intestine, STAT6 causes p38 MAPK inhibition, since STAT6 phosphorylation was still visible during p38 inhibition.

How might STAT6, or the inhibition of p38, contribute to impaired ENaC activity in intestinal cells? Activation of the GR and MR by glucocorticoids or mineralocorticoids, respectively, and the subsequent transcription of target genes, is the most important physiological regulatory

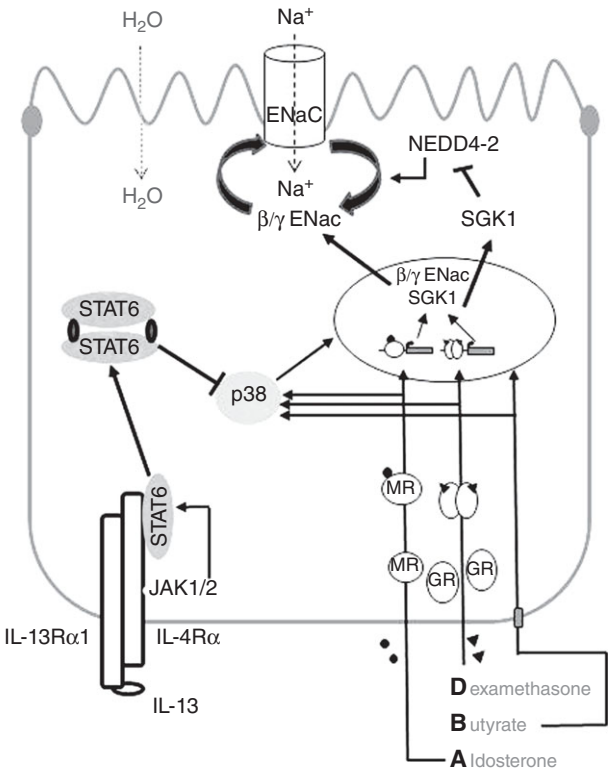

Figure 7. Proposed model for IL-13 action on ENaC-dependent Na^+ absorption
The stimulation with DBA (dexamethasone, butyrate and aldosterone) leads to the up-regulation of β- and γ-ENaC subunit as well as SGK1 expression. On the one hand this is achieved via activation of the p38 MAPK and on the other hand by transactivation of target genes via activated mineralocorticoid receptor (MR) and glucocorticoid receptor (GR). IL-13 binds to the IL-13Rα1/IL-4Rα type-I receptor leading to the activation of JAK1/2. Subsequently, STAT6 is phosphorylated and this in turn leads to the inhibition of the DBA-stimulated activation of p38 MAPK. These signalling events promote the decrease in DBA-stimulated ENaC activity due to reduction of β- and γ-ENaC and/or SGK1 expression. Technical terms used in this figure are also listed in the abbreviations.

process for ENaC induction in the intestine (Epple *et al.* 2000). As also postulated for other STAT proteins (Stocklin *et al.* 1996), Biola *et al.* reported that in murine T-lymphocytes, STAT6 and GR interact to mutually antagonize transactivation (Biola *et al.* 2000). However, our data from reporter gene assays using a representative HRE-driven pMMTV-Luc plasmid argue against the hypothesis of STAT6 affecting GR/MR binding to their respective HREs in intestinal cells. Furthermore, *in silico* analyses (ALLGEN-PROMO, transfac database) failed to reveal STAT6 binding elements within the γ-ENaC promoter fragment (-459 to $+35$), despite an inhibitory effect of IL-13. By contrast, putative binding sites for transcription factors were predicted, which are known targets of p38 MAPK signalling, e.g. Elk1 or CHOP.

Although further studies are required to analyse the precise mechanism by which p38 targets ENaC activity, our data clearly show a significant role of this MAPK in both ENaC activation by DBA and in IL-13-mediated ENaC inhibition. Indeed, there are several reports of p38 MAPK activation leading to upregulation of β- as well as γ-ENaC subunit expression (Itani *et al.* 2003; Niisato *et al.* 2007). Moreover, numerous studies have suggested involvement of p38 MAPK in SGK1 activation (Waldegger *et al.* 2000). For example, Bell and co-workers reported p38 MAPK-dependent stimulation of SGK promoter activity in NMuMg mouse mammary epithelial cells (Bell *et al.* 2000).

In summary, our data show that IL-13 downregulates ENaC-dependent Na^+ absorption in the intestine by coordinated modulation of JAK1/2–STAT6–p38 signalling. This demonstrates that IL-13 in the intestine acts as a potent modulator of transcellular ion transport, in addition to its well-recognized ability to regulate paracellular transport.

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Additional information

Competing interests

The authors declare that they have no conflict of interest associated with this manuscript.

Author contributions

All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed. P.D., J.D.S. and A.F. developed the conception and design of the experiments. P.D. and A.F. performed the experiments and analysed and interpreted the data. P.D. wrote the manuscript. T.B., R.B., C.B., S.K., M.F. and J.D.S. critically reviewed the manuscript. The experiments were performed in the laboratory of the Department of Gastroenterology, Infectious Diseases and Rheumatology; Charité – University Medicine Berlin, Campus Benjamin Franklin, Berlin, Germany.

Funding

This study was supported by Deutsche Forschungsgemeinschaft (DFG) grants Schu 559/10-2 and the Sonnenfeld-Stiftung Berlin.

Acknowledgements

We thank Prof. Geoffrey Sandle (Leeds, UK) for his great help in preparing the manuscript. We acknowledge the excellent technical support of Detlef Sorgenfrei (deceased February 2015).

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Translational perspective

In addition to its well-recognized ability to regulate epithelial barrier function in the intestine, interleukin-13 (IL-13) acts as a potent modulator of transcellular ion transport. It does this by downregulating sodium transport mediated by epithelial sodium channels (ENaC) in the distal large intestine, through a coordinated modulation of JAK1/2, STAT6 and p38–MAP kinase signalling. In healthy colon, basal ENaC activity is low, but is readily increased during significant fluid and electrolyte losses. Thus, during diarrhoeal disorders in which disturbances of the intestinal epithelial barrier results in consecutive leak fluxes of solutes and water, ENaC is stimulated by increased circulating levels of aldosterone to conserve sodium and water. However, this can be hampered by IL-13, for example in ulcerative colitis, a Th2-driven inflammatory bowel disease. A fuller understanding of the mechanisms involved in IL-13-dependent transport and barrier defects may therefore provide new therapeutic strategies for diarrhoeal diseases in which IL-13 plays a key role. It is also feasible that novel multimodal therapeutic approaches may be required in intestinal diseases where pathophysiological changes in epithelial function reflect the simultaneous effects of several proinflammatory cytokines.