Toll-like receptor 5- and lymphotoxin beta receptor-dependent epithelial Ccl20 expression involves the same NF-kappaB binding site but distinct NF-kappaB pathways and dynamics.

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Jean-Claude Sirard [1] **, Arnaud Didierlaurent** [2] **, Delphine Cayet** [1] **, Frédéric Sierro** [3] **, Martin Rumbo** [4 *]

[1] *Interactions cellulaires et moléculaires des bactéries pathogènes avec l'hôte INSERM : U801 , Institut Pasteur de Lille , Université du Droit et de la Santé - Lille II , Institut de Biologie de Lille 1, Rue du Professeur Calmette 59021 LILLE CEDEX,FR*

[2] *Kennedy Institute of Rheumatology imperial College of London , Faculty of Medicine, London,GB*

[3] *Department of Immunology and Inflammation The Garvan Institute of Medical Research , Darlinghurst,AU*

[4] *UNLP, Laboratorio de Investigaciones en el Sistema Inmune Facultad de Ciencias Exactas , La Plata,AR*

* Correspondence should be adressed to: Martin Rumbo <martinr@biol.unlp.edu.ar >

Abstract
Summary

Canonical and alternative NF-κB pathways depend on distinct NF-κB members and regulate expression of different gene subset in inflammatory and steady state conditions, respectively. In intestinal epithelial cells, both pathways control the transcription of the gene coding the CCL20 chemokine. Lymphotoxin β receptor (LTβR) mediates long lasting CCL20 expression whereas Toll-like receptor 5 (TLR5) signals promote inducible and transient activation. Here, we investigated whether the regulation of CCL20 expression involves different promoter sites and NF-κB molecules in response to TLR5 and LTβR stimulation. In epithelial cells, both stimulation required the same promoter regions, especially the NF-κB binding site but involved different NF-κB isoforms: p65/p50 and p52/RelB, for TLR5 and LTβR-dependent activation, respectively. The dynamic of activation and interaction with CCL20 -specific NF-κB site correlated with gene transcription. Similar Ccl20 expression and NF-κB activation was found in the small intestine of mice stimulated with TLR5 and LTβR agonists. In summary, different NF-κB pathways modulate CCL20 transcription by operating on the same NF-κB binding site in the same cell type.

MESH Keywords Active Transport, Cell Nucleus ; Animals ; Base Sequence ; Binding Sites ; Caco-2 Cells ; Cell Line ; Cell Nucleus ; metabolism ; Chemokine CCL20 ; biosynthesis ; genetics ; metabolism ; Flagellin ; pharmacology ; Gene Expression ; drug effects ; Humans ; Lymphotoxin beta Receptor ; agonists ; metabolism ; Mice ; Mice, Inbred C57BL ; Molecular Sequence Data ; NF-kappa B ; metabolism ; Promoter Regions, Genetic ; Protein Binding ; Signal Transduction ; Toll-Like Receptor 5 ; metabolism

Author Keywords NF-kappa-B ; Toll-Like Receptor ; Lymphotoxin beta ; Chemokine ; CCL20

Introduction

Chemokines promote the migration of immune cells under physiological and inflammatory conditions [1]. Homeostatic chemokines are constitutively produced in organs contributing to steady-state leukocyte trafficking and to lymphoid tissue organization. In contrast, the pro-inflammatory chemokines are selectively expressed upon microbial stimulation or tissue injury and recruit immune cells to sites of inflammation. The archetypical example is CXCL8 (IL-8) that is produced by epithelial cells upon pathogen infections [2]. Some chemokines like CCL20 can act as both constitutive/homeostatic and inducible/pro-inflammatory mediator depending on the conditions [3], [4]. The mechanisms governing such dual expression are however not yet understood.

While most chemokines bind several chemokine receptors and most receptors recognize several chemokines, CCL20 interacts specifically with the receptor CCR6 ([1], [3], [4], [5]). CCL20 promotes the recruitment of immature dendritic cells (DC) as well as B cells and activated T cells, thus eliciting adaptive immune responses [6]. Both in humans and mice, CCL20 expression is rather localized within mucosal sites [7], [8], [9], [10], [11]. Along the small intestine, the CCL20 chemokine is permanently produced by follicle-associated epithelium (FAE) contributing to DC positioning in the subepithelial area of Peyer's patches [12], [6], [13]. The constitutive CCL20 expression on FAE likely depends on lymphotoxin β (LTβ) receptor (LTβR) signaling in epithelial cells [14]. Otherwise, microbial signals or cytokines like interleukin 1β (IL-1β), tumor necrosis factor α (TNF α) transiently upregulate CCL20 expression within epithelial cells [15], [16], [17], [18], [10]. We and others showed that Toll-like receptor 5 (TLR5) activation by flagellin stimulates epithelial production of CCL20 and other chemokines like CXCL8 [19], [20], [21], [22]. Both LTβR and TLR5 signaling stimulate NF-κB activation to promote CCL20 transcription.

NF-κB transcription factors form homodimers or heterodimers of five distinct proteins: p50, p52 (p100), p65 (RelA), RelB, and c-Rel that bind the consensus motif $G_{-5}\,G_{-4}\,G_{-3}\,R_{-2}\,N_{-1}\,N_{0}\,Y_{+1}\,Y_{+2}\,C_{+3}\,C_{+4}$ widely spread within mammalian gene promoters [23], [24]. Upon stimulation by pro-inflammatory or microbial signals, the canonical NF-κB p65/p50 binds to NF-κB sites of promoters causing transcriptional activation. Such activation involves the proteolysis of NF-κB-bound IκBα proteins and the nuclear translocation of p65/p50

[24]. NF-κB p65/p50 activity induces almost immediate gene expression, including genes coding repressors, which mediate a negative feedback for early response termination. A second major NF-κB activation pathway, called the non-canonical or alternative pathway involves the NF-κB constituted of p52/RelB dimers. This pathway is triggered by various receptors such as TWEAK-R, CD40 or LTβR [24]. Gene transcription is slower and long-lasting with p52-dependent signals compared to canonical NF-κB [24], [25]. Cell type- and stimulus-specific factors determine the NF-κB combination involved in the transcriptional regulation of a specific subset of genes. How a promoter harboring NF-κB binding motif discriminates the canonical and alternative pathways may depend on (1) relative levels of p65/p50 and p52/RelB levels in steady-state conditions, (2) variability of the NF-κB sites that selectively bind p65/p50 or p52/RelB, (3) specific expression of receptors controlling NF-κB pathways, (4) cell- or stimulus-specific chromatin changes and cooperation with parallel signal transduction pathways and transcription co-factors.

The sequences from the human CCL20 and mouse Ccl20 promoters are highly homologous (Supplementary Figure 1). Particularly, the binding site specific for NF-κB that is proximal to the transcription initiation site (−93 to −82 for human and −98 to −87 for mouse) is conserved and is critical for CCL20 promoter activity in both species [17], [18], [26], [20]. We have previously shown that LTβR signaling in intestinal epithelial cells induces p52 nuclear activity and sustained CCL20 expression [14]. Mutation in the NF-κB site of CCL20 promoter abrogates the LTβ-mediated transcription [14]. In intestinal epithelial cells, TLR5 signaling activates CCL20 transcription through the canonical pathway [20]. The kinetic of CCL20 expression mediated by LTβR is different from that mediated by TLR5 signaling. While TLR5 activation triggers an early and transient increase of CCL20 transcription, LTβR signaling promotes a sustained increase of CCL20 mRNA levels [14], [21]. Here, we further analyzed the contribution of NF-κB binding sites on CCL20 promoter and NF-κB molecules in the regulation of expression in intestinal epithelial cells upon TLR5 or LTβR stimulation. We showed that both stimuli require the same NF-κB site but recruits p65 and p52, respectively. We found that the dynamic of NF-κB recruitment and negative feedback is different according to stimulus. Besides experiments on cell lines, we observed similar recruitment in the small intestine of TLR5- or LTβR-stimulated mice. These data show that in intestinal epithelial cells, different NF-κB pathways can modulate gene expression interacting on the same NF-κB binding site of a specific gene promoter.

Experimental Procedures

Cell Culture

All cell culture products were from Gibco BRL (Rockville, USA). The human intestinal epithelial-like cells T84 and Caco-2 cells (clone 1) and the mouse cell line Iccl2 were maintained as previously described [27], [21]. Briefly, T84 and ICcl2 cells were grown in 50 % DMEM, 50% Ham's F12 medium, 5% FCS and 2 mM L-glutamine. Caco-2 cells were cultured in DMEM with glutamax, 10% FCS, 1% non-essential amino acids and 4 μg/ml transferrine. Cells were grown for 3–7 days to confluency and then treated with medium supplemented with human $LT\alpha_1 \beta_2$ (1000 ng/mL) from R&D (Carlsbad, USA) or agonist antibody specific to human LTβR from R&D (100 ng/mL) or to mouse LTβR (100 ng/mL, clone 4H8 WH2) from Alexis (Switzerland) or flagellin (1000 ng/mL) and harvested at different times. Endotoxin-free flagellin FliC was prepared from Salmonella enterica Serovar Typhimurium ATCC 14028 as previously described [19] or purchased from Alexis. Human or mouse CCL20 levels in cell culture supernatant were determined by ELISA (R&D Systems, USA) following supplier's instructions.

Cell transfections and luciferase assays

Previously, the CCL20 promoter region (about 1.6 kb) was cloned into the firefly luciferase reporter pGL-3 basic vector (Promega, USA), giving rise to Δ1451 plasmid, and site directed mutagenesis using the primer 5′ GGGCCAGTTGATCAATgatgagaattCCATGTGGCAACACGC 3′ (mutated nucleotides are written in small letters) was performed to mutate the putative CCL20 NF-κB binding [14]. Truncated promoters were generated using the Δ1451 plasmid and the double-stranded nested deletion kit (Pharmacia, Sweden). Epithelial cells were transfected for 12 h with the CCL20 reporter plasmids and the normalizing pRL-TK plasmid coding Renilla luciferase (Promega) using Lipofectin® (Invitrogen, Carlsbad, USA). Fresh cultured medium was added for 48 h and the cells were stimulated for 6 to 9 h as described above. Firefly luciferase activity was measured and normalized to Renilla luciferase activity using the Dual luciferase® assay (Promega). Relative luminescence (RLU) was normalized as luciferase RLU (RLU-L) with Renilla RLU (RLU-R) and the variation in luciferase activity was calculated as follows $[(\text{RLU-L}_{\text{treated}}/\text{RLU-R}_{\text{treated}})/(\text{RLU-L}_{\text{mock}}/\text{RLU-R}_{\text{mock}})]$ using as mock condition cells transfected with full length promoter fusion that were not activated by any stimulus.

Nuclear extract analysis

At indicated times after stimulation, cells were washed with cold PBS containing phosphatase inhibitors and nuclear proteins were obtained using the Nuclear Extract kit from Active Motif (Carlsbad, USA) following manufacturer instructions. Protein concentration of the extracts was measured using the BCA Assay (Pierce, Rockford, USA). DNA binding activity of NF-κB proteins within nuclear extracts was assessed using the TransAM™ NF-κB family kit (Active Motif). Briefly, 500 ng of nuclear extract were incubated 1h RT into a 96-well plate coated with an oligonucleotide bearing an NF-κB consensus binding. After washing, plates were incubated with rabbit primary antibodies specific for human p65, p50, p52 or RelB for 1h at RT. The anti-p65 and anti-p52 antibodies that are crossreactive with

mice counterparts were used in experiments involving ICcl-2 cells or animal samples. Revelation was done with HRP-conjugated anti-rabbit IgG and TMB as substrate. Absorbance was measured at 450 nm in a microplate spectrophotometer. Competition was performed with the TransAM™ NF-κB family kit oligonucleotide, purified double strand DNA bearing the CCL20 wild type promoter sequence (5′GGGCCAGTTGATCAATggggaaaaccccATGTGGCAACACGC3′) or a NF-κB mutant variant (5′ GGGCCAGTTGATCAATgctgagaattccATGTGGCAACACGC3′). To generate double strand DNA, sense and antisense oligonucleotides were mixed in TE buffer (10mM Tris, 1 mM EDTA) at a 100 mM concentration, heated at 95°C for 5 min and hybridized by equilibration to RT. Homogeneity of oligonucleotide duplex was assessed by PAGE.

In vivo experiments

C57BL/6 mice were strain from Charles River Laboratories (France). (C57 BL/6J 5 CBA/J)F1 harboring a transcriptional fusion between promoter containing NF-κB sites from the Igκ light chain and the firefly luciferase (3x-κB-luc or NF-κB-luc) were bred in our animal facility [28]. Animals were maintained in a specific pathogen-free facility in an accredited establishment (#A59107; Institut Pasteur de Lille). All experiments complied with current national and institutional regulations and ethical guidelines. Mice were stimulated by intraperitoneal (i.p.) injection of PBS, flagellin (10μg), agonistic anti-LTβR antibody or control antibody (10μg) and sacrificed at indicated times. Luciferase activity was measured on intestinal homogenates of NF-κB-luc mice using Luciferase Assay System (Promega) and normalized to protein content measured using Bradford assay (Pierce). For nuclear extract preparation, small intestine were rinsed with ice chilled PBS, Peyer's Patches were removed and mucosal layer scrapped gently with a scalpel after addition of lysis buffer supplemented with protease and phosphatase inhibitor cocktail from Active Motif. Nuclear extracts were obtained and assayed as described above. Mouse CCL20 levels were determined by ELISA (R&D Systems, USA) on intestinal tissues homogenized in T-PER Tissue Protein Extraction Reagent (Pierce) supplemented with Complete protease inhibitor (Roche). Chemokine levels were also normalized to the total protein content.

In situ hybridization

It was performed as previously described [14]. Briefly Ccl20 -specific ^{35}S -labeled riboprobes were synthesized from a plasmid vector pKS containing full coding region. Paraformaldehyde-fixed frozen sections were protease-treated, washed and the slides were hybridized at 45°C overnight with a labeled probe at 2×10^6 cpm/10μL. After RNase treatment and washes, the radioactive probe bound to tissue section mRNA specific for Ccl20 was detected by 2–4 weeks exposure to emulsion autoradiography using NTB-2 emulsion (Eastman Kodak Co.). As negative control, antisense probe was used.

Laser microdissection

Small intestine were rinsed with ice chilled PBS and fixed overnight at 4°C in a solution containing 300g sucrose, 5g $ZnCl_2$, 6g $Zn(O_2CCH_3)_2(H_2O)_2$, 0.1g $Ca(O_2CCH_3)_2$ in 1L of 0.1M Tris-HCl pH7.4. Tissues were subsequently frozen embedded in OCT and 20μm sections were mounted on Leica slides for microdissection (Leica Microsystems, Wezlar, Germany), stained with Mayer's Hemalun and processed as previously described [21]. Using a LMD microscope AS LMD (Leica Microsystems), epithelial layer of intestine was cut out from slides and samples were collected into RNA lysis buffer.

Quantitative real-time RT-PCR

Total RNA was extracted with the Nucleospin RNA II kit (Macherey Nagel, Germany) and reverse-transcribed with the High-Capacity cDNA Archive Kit (Applied Biosystems, USA). The resulting cDNA was amplified using SYBR Green-based real-time PCR (Applied Biosystems). The specific primers are CGTCATCCATGGCGAACTG/GCTTCTTTGCAGCTCCTTCGT (ActB , coding for mouse β -actin), TTTTGGGATGGAATTGGACAC/TGCAGGTGAAGCCTTCAACC (mouse Ccl20), AGACTGCCCGCAAATCGAC/CTTGCGAGCGGCTTTTGTA (H3F3A , coding for human histone 3.3), and CCAAGAGTTTGCTCCTGGCT/TGCTTGCTGCTTCTGATTCG (human CCL20). Relative mRNA levels ($2^{-\Delta\Delta Ct}$) were determined by comparing (a) the PCR cycle thresholds (Ct) for Ccl20 and ActB or H3F3A for mouse and human, respectively (ΔCt) and (b) ΔCt values for treated and control groups (ΔΔCt) as described (19).

Statistical analysis

Statistical differences were analyzed using the Mann-Whitney and Student t tests and were considered to be significant for p values <0.05 . Unless otherwise specified, results are expressed as arithmetic means ± standard deviation.

Results

Similar CCL20 promoter elements are required for TLR5 and LTβR signaling

Recent studies found that CCL20 transcription is upregulated by flagellin-TLR5 and LTβ-LTβR signaling in the human intestinal epithelial cell lines: T84 and Caco-2 [21], [19]. The transcriptional fusion Δ1451 between the genomic DNA fragment encompassing 1451

bp upstream and 38 bp of human CCL20 first exon and luciferase gene (luc) was previously constructed [14] (Figure 1). The promoter harbors several putative transcription factor binding sites including NF-κB (Figure 1a). In order to assess their contribution, we used the Δ 1451 fusion mutated in the NF-κB binding site and we generated serial deletions of the promoter to positions −214, −195, −173, −163, −117 or −102 (giving rise to constructs Δ214, Δ195, Δ173, Δ163, Δ117, and Δ102 respectively) (Figure 1a). Luciferase activity was assessed in T84 cells after transient transfection and TLR5 and LTβR stimulation, using flagellin and agonistic anti-LTβ antibodies, respectively (Figure 1b–c). The Δ1451 promoter was sufficient to promote 6-fold upregulation of fusion activity for both stimuli. Similar results were obtained with fusions Δ214, Δ195, Δ173, and Δ163 (Figure 1b–c and data not shown). These data showed that the CCL20 promoter activity requires at least the 163bp region upstream of transcription initiation site. In contrast, the fusions Δ117 and Δ102 were totally unable to respond to signals meaning that essential sites for CCL20 transcription are located between −163 and −117. Kwon et al. [17] showed that the site from −143 to −154 is critical for the Ets-like transcription factor ESE-1 activity and CCL20 expression in epithelial cells. This may explain the results obtained with our truncated constructs. Similarly, mutation on the NF-κB binding site (−93 to −82 bp) impaired activation of transcriptional fusion by both LTβ and flagellin, thereby confirming the key role of NF-κB for pro-inflammatory or homeostatic CCL20 expression (Figure 1b–c)[14 , 17 , 18]. Similar results were obtained for flagellin stimulation in the Caco-2 cells (data not shown). Finally, the mutation of the NF-κB binding motif and any promoter deletions that impaired response also affected the basal CCL20 promoter activity (Figure 1b–c). In conclusion, activation of transcription by pro-inflammatory TLR5 or homeostatic LTβR signaling requires the same binding sites for NF-κB and additional co-factors in the CCL20 promoter.

Different NF-κB members are translocated to the nucleus upon TLR5 and LTβR signaling

The kinetic and the magnitude of the NF-κB-dependent transcriptional responses depend on the cell type and are different upon TLRs and LTβR signaling. We studied CCL20 transcription after LTβR and TLR5 stimulation of intestinal epithelial cells. T84 cells are responsive to both flagellin and LTβ whereas Caco-2 cells exclusively respond to flagellin [21]. We also used the mouse cell line ICcl-2 that responds to LTβ but poorly to flagellin [29]. Whereas TLR5 signaling activated an early and transient CCL20 gene transcription, LTβ R stimulation promoted sustained expression (Figure 2a). In both cases, transcriptional response correlated with CCL20 protein production as measured in the cell culture supernatant (Figure 2b and c). T84 cell line showed a higher basal expression of CCL20 protein however a significant increase in CCL20 levels could be detected after either flagellin or LTβ stimulation.

We next determined the nature of NF-κB members activated in epithelial cells by LTβR and TLR5 stimulation using an ELISA that detects the specific binding of transcription factors to DNA. In brief, nuclear extracts from mock or treated cells were incubated with immobilized NF-κB oligonucleotides and the binding of NF-κB was detected using specific antibodies. The kinetics of nuclear translocation of p50 (p105), p52 (p100), p65 and RelB upon TLR5 or LTβR signaling were first analyzed. The effect of flagellin on T84 and Caco-2 cells, and anti-LTβR stimulation on T84 and ICcl2 cells is depicted in Figure 3 . In Caco-2 cells, flagellin triggered an early translocation of p50 as observed at 30 min. Then, p50 steady state levels were totally restored at 8h (Figure 3a). Similar results were observed for p65 (Figure 3b), whereas no changes were observed for nuclear levels of p52 upon flagellin stimulation on this cell line (Figure 3c). A transient increase of nuclear p50 and p65 was also observed in T84 cells (Figure 3d–e), even though their basal levels were high in untreated cells, in concordance with higher levels of basal CCL20 production. Moreover, in coincidence with Caco-2 results, flagellin did not cause any translocation of RelB and p52 in this cell line (Figure 3f–g). As expected, these data support the specific activation of canonical pathway by TLR5 signaling. In contrast, after LTβR activation, RelB and p52 were detected in nuclear extracts of T84 cells (Figure 3j–k). However, the translocation was delayed compared to TLR5 data and long-lasting since RelB and p52 were present from 8 to 24h after activation, as previously described [14]. Similar features were found for ICcl2 mouse cells (Figure 3l–m). Upon LTβR treatment, a faint activation of p65 and p50 was consistently observed at early and late time points. These data strengthened previous observations that LTβR triggers preferentially the alternative NF-κB pathway in epithelial cells. The NF-κB activation pattern and kinetics observed is coincident with the kinetics of CCL20 expression (Figure 2). In summary, the signaling pathway influences both the type and the dynamic of intra-nuclear NF-κB recruitment, thereby modulating differentially CCL20 transcription.

Specific binding of p65 and p52 to the CCL20 promoter proximal NF-κB binding site

In ELISA assays, the immobilized oligonucleotides contain the consensus sequence: GGGACTTTCC that binds p50/p65, p52/RelB as well as other NF-κB family member combination. Although NF-κB sites have usually little selectivity for a given NF-κB species, there are some reports supporting this mechanisms [30]. The NF-κB binding site of CCL20 promoter: GGGAAAACCC, was shown to be essential for both TLR5 or LTβR signaling (Figure 1) [14]. To determine whether this site is specific for p65 and/or p52, we performed competition using CCL20 -specific oligonucleotides that span the NF-κB binding site of CCL20 promoter or mutant oligonucleotides (Figure 4 and data not shown). We selected the conditions resulting in maximal nuclear levels of p50/p65 and p52/RelB translocation after stimulation with flagellin and LTβ (30 min and 24h, respectively). As shown, the CCL20 -specific oligonucleotides efficiently inhibited the binding of the p50, p52, p65 and RelB, independently of the stimulation. In contrast, the mutant oligonucleotide did not promote any competition of the immobilized NF-κB site. These results suggest that the different NF-κB combinations interact with CCL20 promoter at the same binding site.

Intestinal Ccl20 expression upon TLR5 or LTβR stimulation correlates with differential recruitment of NF-κB members

To define the physiological relevance of NF-κB binding, we analyzed Ccl20 expression and Ccl20 promoter activity in vivo upon LTβ R and TLR5 stimulation (Figure 5). As previously observed for LTβR signaling [14], intraperitoneal injection of flagellin induced Ccl20 expression in the intestinal absorptive epithelium layer of the ileum (Figure 5a). We also detected the constitutive Ccl20 transcription within the FAE, a process likely LTβR-dependent. The expression kinetics upon TLR5 or LTβR signaling in vivo was similar to the one observed on intestinal epithelial cell lines. Using laser-microdissected epithelium, we found that flagellin induced early and transient Ccl20 expression while anti-LTβR treatment provoked early and sustained expression (Figure 5b). For both stimuli, the transcriptional induction was also correlated with a rise in CCL20 protein levels of treated intestines, although higher interindividual variability was observed (Figure 5c). Using NF-κB-luc reporter mice, we found that flagellin treatment activated NF-κB throughout the small intestine (Supplementary Figure 2). We next assessed NF-κB activity using nuclear extracts obtained from scrapped intestinal mucosal layers (Figure 5d–e). Coincident with the kinetics of Ccl20 expression, flagellin stimulation induced an early nuclear p65 translocation whereas anti-LTβR treatment promoted p52 translocation after 8h. Moreover, complete inhibition of p65 and p52 activities was obtained by competition with the oligonucleotide harboring a native CCL20 NF-κB site while the mutant counterpart had no effect. In summary, we have observed in intestinal mucosa the same kinetics of CCL20 expression and NFκB kinetics and differential activation than observed in intestinal epithelial cell lines. Besides, p65 and p52 can bind to the native NFκB binding site of Ccl20 promoter.

Discussion

The epithelial production of CCL20 chemokine in the small intestine plays a central role in adaptive immune responses by a CCR6-dependent recruitment of immature dendritic cells [19]. LTβR signaling triggers CCL20 production on intestinal epithelial cells, being probably involved in long-lasting CCL20 production in FAE of Peyer's patches [14]. On the other hand, infectious or inflammatory stimuli like TLR5 activation by flagellin promote transient CCL20 transcription [19]. Our study aimed to analyze the CCL20 promoter sites and the NF-κB molecules involved in stimulus-specific gene regulation. Our results indicate that the region containing the 166 nucleotides upstream the transcription initiation start, are required for both receptors. This region contain several sites that have been shown to be important for CCL20 regulation such as ETS (−143−151), AP-1 (−113 to −120) and NF-kB (−82 to −93) binding sites [17]. We focused on the proximal NF-κB binding site since we observed that site-directed mutagenesis of this site abolishes inducibility of CCL20 promoter either by proinflammatory signals as well as by LTβR stimulation. The NF-κ B site is critical for TNFα and IL-1β -induced CCL20 expression on intestinal cells through a rapid and transient recruitment of p65/p50 [10 , 18],[17]. The NF-κB site (G_{-93} GGGAAAACCCC$_{-82}$) of CCL20 promoter recruit different NF-κB isoforms: p65/p50 and p52/RelB, for TLR5 and LTβR-dependent activation, respectively (Figures 3 –5). This pattern is characteristic of canonical and alternative NF-κB pathways. The kinetic of NF-κB translocation to nucleus correlated with that of CCL20 transcription, i.e. transient and long-lasting for TLR5 and LTβR, respectively. CCL20 mRNA stability in the presence of transcription inhibitors indicated a high turnover (data not shown), thereby arguing that the signaling does not specifically affect stability as shown for other genes triggered by proinflammatory stimulation [31]. These observations highlight the importance of the transcriptional control in CCL20 expression. Using animal model, we found that upon LTβ or flagellin treatment, dynamic of Ccl20 transcription and NF-κB recruitment in the small intestine correlates, in concordance with the in vitro evidence. Therefore, our data first report that both canonical and alternative NF-κB pathways modulate CCL20 transcription within intestinal epithelial cells in a stimulus-specific manner by operating on the same NF-κB site.

Using different intestinal epithelial cells lines, we studied the CCL20 promoter regions required for expression. Reporter constructs under the control of truncated versions of CCL20 promoter allowed to determine that TLR5- or LTβR-mediated CCL20 expression depend on the same promoter motifs (Figure 1). TLR5 stimulation promote similar dynamic of NF-κB translocation and gene expression. In splenocytes, LTβR signaling activates a biphasic NF-κB response; the canonical NF-κB pathway is first switched on with fast nuclear translocation of p65/p50 followed by the alternative NF-κB pathway that induces a sustained translocation of RelB/p52 [32]. In epithelial cells, LTβR stimulates similar activation kinetics of the canonical alternative pathway [14]. Here, we further investigated the activity of NF-κB family members on CCL20 promoter by using a solid phase transcription factor binding assay that combines high sensitivity and flexibility to perform competitive assays [33]. The kinetics of NF-κB nuclear translocation upon flagellin or anti-LTβR stimulation observed was coincident with previous western blot analysis [14]. The binding of the NF-κB members to a consensus oligonucleotide was specifically abolished by competition with an excess of 42-mer oligonucleotide bearing the human CCL20 promoter NF-κB binding site, meaning that either p65/p50 or RelB/p52 are binding to the same NF-κB binding element on human CCL20 promoter (Figures 4 –5). Like experiments based on the CCL20-luc fusions, the competition in NF-κB binding assays was abrogated by introducing similar mutations in the CCL20 -specific NF-κB binding sequence.

The role of NF-κB signaling pathway in the homeostasis of intestinal mucosa and the orchestration of immune responses has been highlighted by the use of tissue specific knock-out mice strains [34], [35]. Using NF-κB-luc reporter mice, we showed NF-κB activation along the gastrointestinal tract upon flagellin intraperitoneal stimulation. We observed by in situ hybridization and laser microdissection that such treatment elicits a transient epithelium-specific Ccl20 transcription, as described in vitro (Figure 5). Although several cell types express TLR5 along the gastrointestinal tract [36], [37] upregulation of Ccl20 expression upon systemic flagellin treatment was

specifically restricted to epithelium. The presence of binding sites for epithelium-specific transcription factors as ESE-1 within Ccl20 promoter [17] may account for this tropism. LTβR signaling produces a sustained upregulation of Ccl20 expression in intestinal epithelium ([14] and Figure 5). In intestinal cell lines, maximal Ccl20 mRNA levels and CCL20 protein production were systematically consecutive to the peak of nuclear translocation of NF-κB members. Since NF-κB binding site in human CCL20 and mice Ccl20 promoters are identical, we inferred that in vivo NF-κB members will bind to the same site in the CCL20 promoter on intestinal epithelial cells. We found that intestinal nuclear extracts isolated from animals treated with TLR5 or LTβR agonist promote the binding of p65 and p52, respectively, to 42-mer oligonucleotide bearing the human CCL20 promoter NF-κB binding site.

The alternative NF-κB pathway is stimulated by different receptors such as CD40, TWEAK, BAFF-R and LTβ-R, depending on stimulation and cell type [38], [39], [40]. Production of homeostatic chemokines such as CXCL12, CXCL13, CCL19 and CCL21 is regulated by LTβR signaling and transcriptional activation by RelB/p52 binding to their respective promoters [32], [30]. However in these conditions, p65 binding to those promoters or upregulation of expression of these homeostatic chemokines was never observed after activation of the canonical NF-κB pathway. This indicates that structural differences at the promoter level may selectively enhance the binding of a particular NF-κB combination.

Using random site selection to identify p52/RelB binding sites, Britanova et al. [41] showed that any DNA motif that binds the heterodimer p52/RelB in vitro, also binds p50/RelA. The CCL20 promoter NF-κB binding site was not retrieved in their study. This finding suggests that p52/RelB-specific binding sites are unusual, supporting the idea that degenerate binding of NF-κB members to NF-κB sites is the rule [23]. Our results show that in the intestinal epithelial CCL20 response, the presence of intact NF-κB binding site proximal to the transcription initiation site is critical for expression induction either under canonical or alternative NF-κB activation. Moreover, this single NF-κB site can bind both alternative and canonical NF-κB mediators as was shown for other cases [41], [23]. Noteworthy, slight modifications of NF-κB binding sites and surrounding motifs may have dramatic effect on the fine tuning of the transcriptional response [42] outlining the importance of additional factors in the regulation of NF-κB-dependent gene expression.

The major role of CCL20 in homeostatic immunity and inflammation may have modeled its promoter to respond to different NF-κB members and NF-κB-dependent signals in a unique cell type. To achieve immune functions of CCL20, the homeostatic signal promote long-lasting processes whereas the inflammatory signal favors transient functional activation of the epithelium. Further investigations are expected to improve our understanding on how the epithelial cell fine tune the overall transcriptional response to canonical or alternative pathways.

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Figure 1

Human CCL20 promoter regions required for TLR5- anf LTβR-mediated expression

(a) Human CCL20 promoter organization. The sequence of the promoter cloned into luciferase reporter plasmid Δ1451 is shown. Bases are numbered according to relative position to transcription initiation site (+1). Binding sites for transcription factors in the 250 bp proximal to +1 are marked. Positions of nested deletions within the promoter transcriptional fusion are indicated by arrows. (b, c) Transcriptional fusion activity of upon stimulation. T84 cells were transfected with a plasmid containing firefly luciferase gene controlled by the various CCL20 promoters and a normalizing plasmid coding Renilla luciferase. Cells were stimulated 48h after transfection with flagellin 1 μg/mL (b) or LTβ 1μg/mL (c). When indicated, the reporter construct harbors a mutated NF-κB binding motif (GGGAAAACCCC → CTGAGAATTCC). Luciferase activity is expressed relative to the Δ1451 reporter fusion in unstimulated conditions. Renilla luciferase was used to normalize the transfection efficiency. Results are the average of at least two independent experiments.

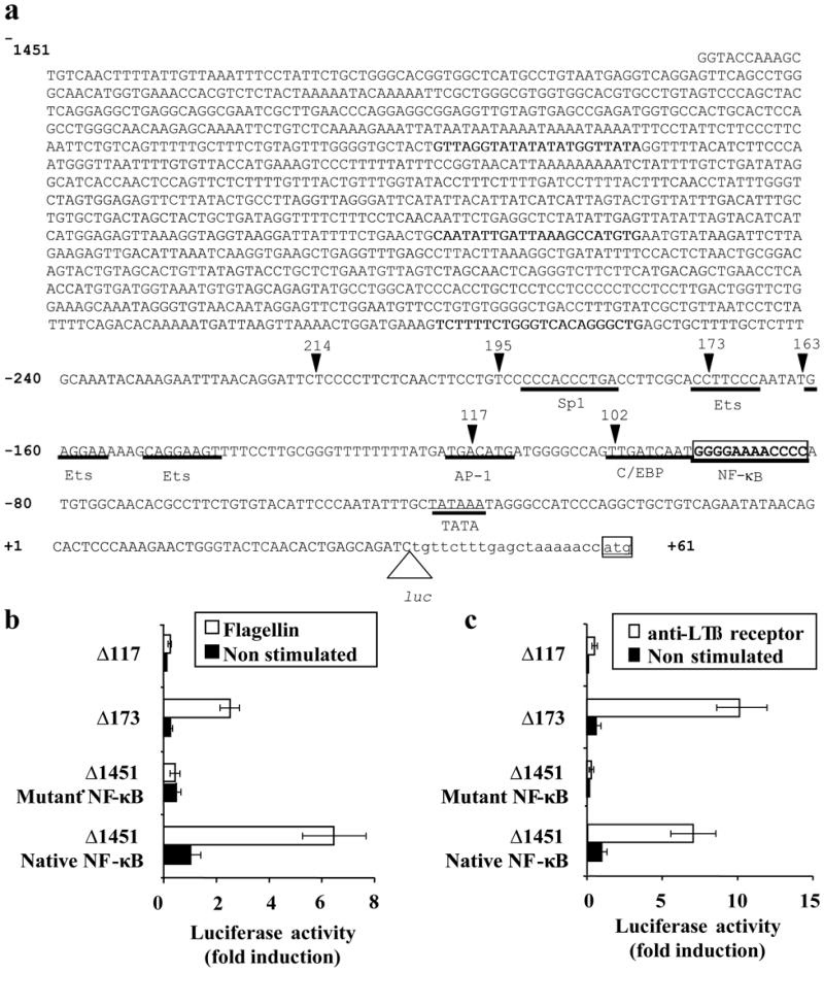

Figure 2

Kinetics of CCL20 expression upon upon flagellin or LTβ stimulation

Intestinal epithelial cells (T84 or Caco-2) were stimulated with either flagellin 1 μg/ml or LTβ (1μg/mL) or LTβR-specific agonist antibody (0.1 μg/mL) and lysed at different times as indicated. CCL20 -specific mRNA levels were measured by RT-qPCR and normalized to levels in untreated cells (a). One representative experiment out of two is shown. CCL20 levels were determined by ELISA in the culture supernatants after 24h stimulation. One representative experiment out of two is shown for T84 cells (b) and Caco-2 cells (c). Levels of significance for paired Student T test: * p <0.02; **p <0.005; *** p <0.002.

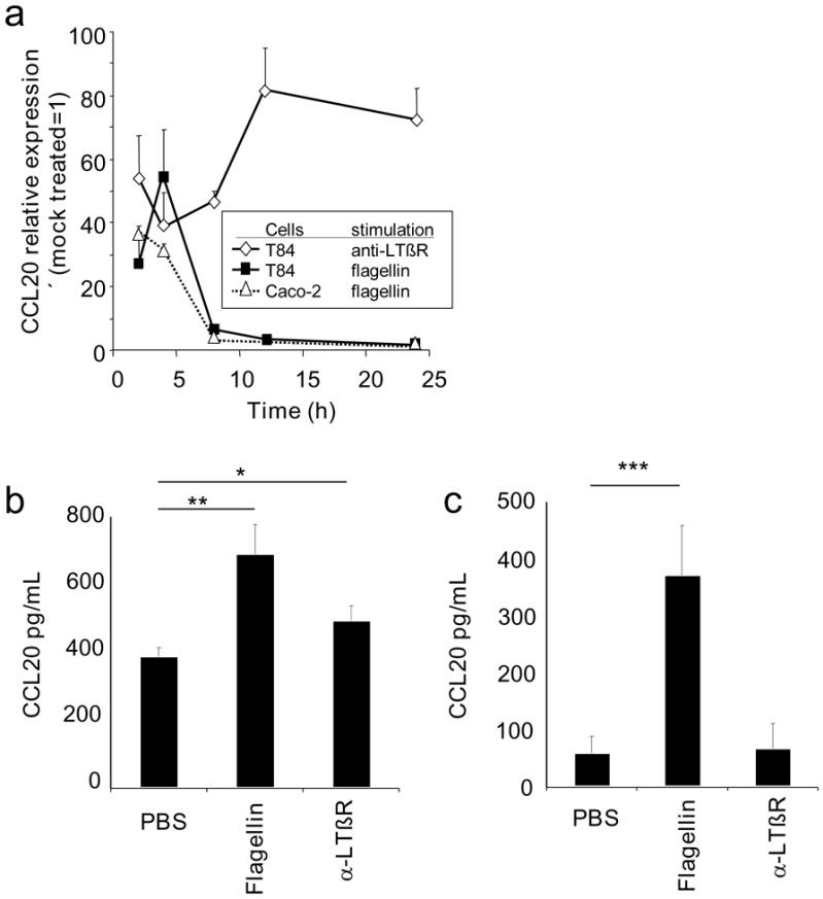

Figure 3

NF-κB members recruited to nucleus upon TLR5 or LTβR stimulation

Intestinal epithelial cells were stimulated with PBS (mock condition), flagellin (1μg/mL), LTβ (1μg/mL) or LTβR-specific agonist antibody (0.1 μg/mL). Nuclear extracts were prepared at indicated times and used to test the presence of different NF-κB family members in a solid phase transcription factor binding assay. The NF-κB binding was detected using antibodies specific for p65 and p52. Nuclear extracts from Raji B cells were used to normalize the OD values in the different assays. Results are expressed as normalized OD obtained after immunoenzymatic development. One representative experiment out of three is shown for human Caco-2 cells upon flagellin stimulation (upper panel, ac), human T84 cells upon flagellin stimulation (middle upper panel, d–g), T84 cells upon LTβR stimulation (middle lower panel h–k) and mouse ICcl2 cells upon LTβR stimulation (lower panel, l and m).

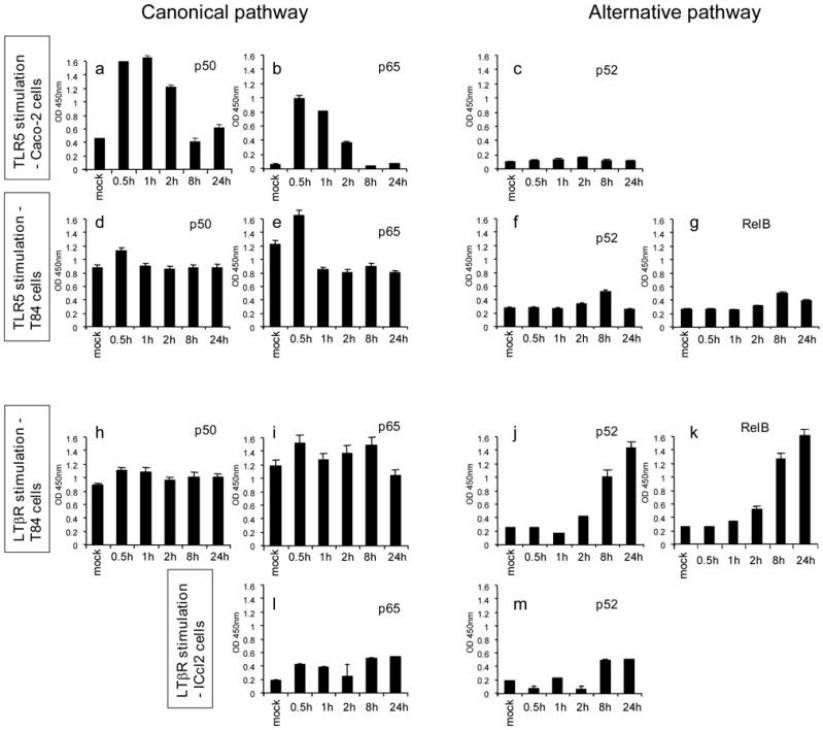

Figure 4

Interaction of NF-κB members with the CCL20 -specific NF-κB binding site

Nuclear extracts from T84 cells that were activated either with flagellin for 30min or LTβR signaling for 8h were selected to assess the competition by NF-κB motifs of CCL20 promoter. Synthetic 42-mer oligonucleotide bearing the human CCL20 promoter sequence encompassing the NF-κB binding site (GGGAAAACCCC) indicated as “Native CCL20 NF-κB” was used as free competitor. Competition was also performed with the oligonucleotide “Mutated CCL20 NF-κB” that contains point mutations within the NF-κB motif (CTGAGAATTCC). Nuclear extracts were incubated in the plate alone or in the presence the competitor oligonucleotide (10 μM). The NF-κB binding was detected by a solid phase transcription factor binding assay ELISA using antibodies specific for p50 (a), p65 (b), RelB (c) and p52 (d). Results are expressed as OD obtained after immunoenzymatic development. Results are the average of two independent experiments in triplicate.

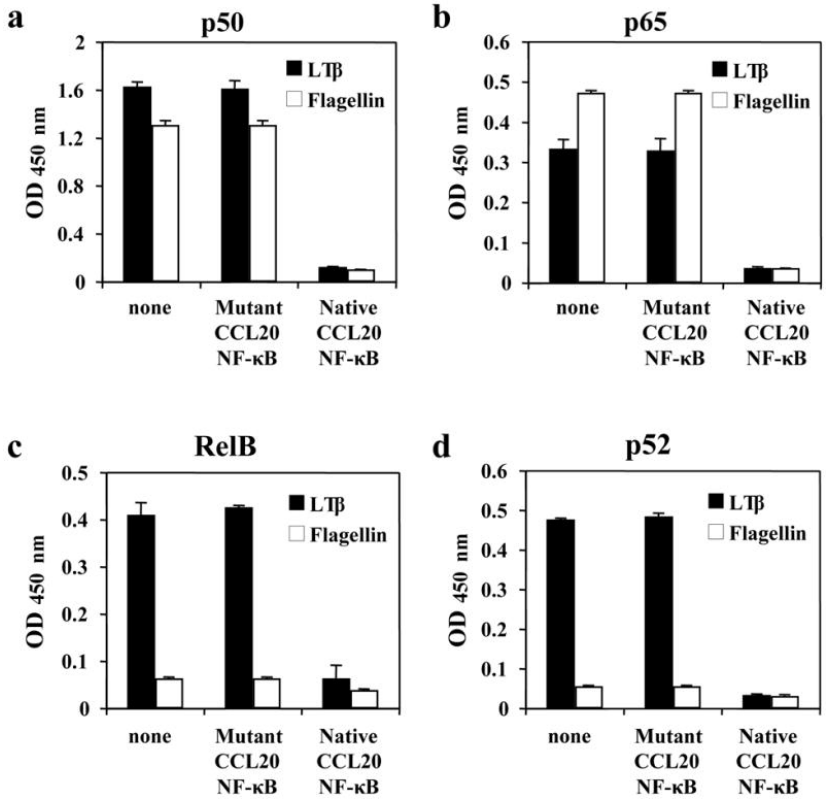

Figure 5

Epithelial Ccl20 expression and NF-κB recruitment in TLR5- and LTβR-stimulated mice

C57BL/6 mice were injected intraperitoneally with PBS, flagellin (10μg), LTβR-specific agonist antibody (10μg) or Ovalbumin (10μg) as a negative control and the small intestine was sampled at indicated times. (a) Flagellin-stimulated Ccl20 expression in epithelium. Intestinal tissues (ileum) were sampled 2h after injection of flagellin and tissue sections were processed for in situ hybridization with Ccl20 -specific anti-sense probe. Ccl20 expression was determined in segments containing Peyer's patches or without PP not. Mock (Ovalbumin) treatment reveals expression in FAE as described previously. Flagellin-stimulated Ccl20 transcription in villous epithelium. (b) Ccl20 expression on intestinal epithelium upon TLR5- or LTβR-stimulation. At indicated times, proximal intestine was sampled and frozen tissue sections were stained and processed by laser dissection microscopy to capture epithelial cell layer. Ccl20 -specific mRNA levels were measured by RT-qPCR and normalized to levels in untreated animals (arbitrary value 1). Results represent the mean of three mice for each condition. (c) CCL20 protein levels in stimulated tissues. Intestinal tissues were sampled and homogeneized after 2h flagellin stimulation or after 8h anti-LT βR i.p. treatment. CCL20 protein levels were determined by ELISA and normalized to the total protein content of tissues. One representative experiment out of two is depicted (d, e) NF-κB recruitment in intestinal mucosa upon TLR5- or LTβR-stimulation. At indicated times, animal' s intestines were sampled and the intestinal mucosa was scrapped on the luminal part to immediately prepare nuclear extracts. The NF-κB binding was detected by a solid phase transcription factor binding assay ELISA using antibodies specific for p52 (c) or p65 (d). Synthetic 42-mer oligonucleotide bearing the human CCL20 promoter sequence encompassing the NF-κB binding site (GGGAAAACCCC) indicated as "Native NF-κB" was used as free competitor. Competition was also performed with the oligonucleotide "Mutated NF-κB" that contains point mutations within the NF-κB motif (CTGAGAATTCC). Nuclear extracts were incubated alone or in the presence the competitor oligonucleotide as described in Figure 4 . Results are expressed as OD obtained after immunoenzymatic development. Results are the average of two independent mice in triplicate and are representative of 2 experiments.