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Anti-Inflammatory Effect of *Lactobacillus casei* on *Shigella*-Infected Human Intestinal Epithelial Cells[1]

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Shigella **invades the human intestinal mucosa, thus causing bacillary dysentery, an acute recto-colitis responsible for lethal complications, mostly in infants and toddlers. Conversely, commensal bacteria live in a mutualistic relationship with the intestinal mucosa that is characterized by homeostatic control of innate responses, thereby contributing to tolerance to the flora. Cross-talk established between commensals and the intestinal epithelium mediate this active process, the mechanisms of which remain largely uncharacterized. Probiotics such as *Lactobacillus casei* belong to a subclass of these commensals that modulate mucosal innate responses and possibly display anti-inflammatory properties. We analyzed whether *L. casei* could attenuate the pro-inflammatory signaling induced by *Shigella flexneri* after invasion of the epithelial lining. Cultured epithelial cells were infected with *L. casei*, followed by a challenge with *S. flexneri*. Using macroarray DNA chips, we observed that *L. casei* down-regulated the transcription of a number of genes encoding pro-inflammatory effectors such as cytokines and chemokines and adherence molecules induced by invasive *S. flexneri*. This resulted in an anti-inflammatory effect that appeared mediated by the inhibition of the NF-κB pathway, particularly through stabilization of I-κBα. In a time-course experiment using GeneChip hybridization analysis, the expression of many genes involved in ubiquitination and proteasome processes were modulated during *L. casei* treatment. Thus, *L. casei* has developed a sophisticated means to maintain intestinal homeostasis through a process that involves manipulation of the ubiquitin/proteasome pathway upstream of I-κBα.** ***The Journal of Immunology,*** **2006, 176: 1228–1237.**

More than 400 species of bacteria are present in the human gut, ranging from 10^5 to 10^7 CFU/g of intestinal contents in the jejunum to 10^{11} to 10^{12} CFU/g in the colon (1). To maintain the homeostatic mechanisms responsible for tolerance of the intestinal mucosa vis-a-vis its commensal flora, and at the same time to permit the development of an innate response to pathogenic bacteria, intestinal epithelial cells (IECs),[4] which are in close contact with luminal bacteria, are expected to play a key regulatory role (2, 3). Intestinal homeostasis has been shown to be under the control of TLRs. Indeed, mice deficient in TLR2, TLR4, or the adaptor molecule MyD88 are more prone to develop intestinal inflammation after dextran sodium sulfate-induced colitis (4). Tolerance to the commensal flora is similarly dysregulated in the case of inflammatory bowel disease (IBD) (5).

Lactobacilli and Bifidobacteria are natural components of the colonic microbiota, and as probiotic agents, they have been tested in the prevention and treatment of IBD (6, 7). A strain of *Lactobacillus casei*, DN-114 001, has been shown to decrease the secretion of TNF-α from the inflammed ileum of Crohn's disease patients (8, 9). The beneficial effect of probiotics is proposed to be due, at least in part, to interference with the innate immune system and possibly the orientation of adaptive immunity. Indeed, the probiotic preparation VSL#3 (a mixture of eight different Gram-positive bacteria) as well as different lactobacilli strains were shown to increase production of the anti-inflammatory cytokine IL-10 by dendritic cells (10, 11). This anti-inflammatory effect was also observed using DNA extracted from these probiotics; VSL#3 DNA was able to reduce IL-8 production by epithelial cells exposed to pro-inflammatory stimuli via a mechanism involving I-κB stabilization (12). Using a nonvirulent *Salmonella* strain, Neish and colleagues showed that regulation of epithelial responses occurs by inhibition of I-κBα ubiquitination (13), which is likely mediated by the *Salmonella*-secreted protein AvrA (14). Finally, regulation of multiple intestinal functions, such as nutrient absorption, mucosal barrier enhancement, and angiogenesis, is also modulated by the commensal *Bacteroides thetaiotaomicron* (15, 16).

Shigella flexneri, an entero-invasive, Gram-negative bacterium, causes the inflammatory destruction of the intestinal epithelium (17), leading to bacillary dysentery, an acute recto-colitis causing lethal complications, mostly in infants and toddlers in the most impoverished areas of the planet. The annual mortality attributed to bacillary dysentery is about one million. The mechanisms leading to acute mucosal inflammation need to be understood to develop efficient methods of prevention and cure. A number of studies have demonstrated that *S. flexneri* infection leads to the

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[4] Abbreviations used in this paper: IEC, intestinal epithelial cell; IBD, inflammatory bowel disease; MRS, Mann-Rogosa-Sharpe; MOI, multiplicity of infection.

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induction of several markers of acute inflammation, such as the chemokines IL-8 and CCL20. These pro-inflammatory genes are under the control of the NF-κB pathway (18), and recently, the mechanism of NF-κB activation in *Shigella*-infected cells has been uncovered. Indeed, our group has demonstrated that after infection, epithelial cells sense the presence of invading *S. flexneri* through Nod1, an intracellular pattern recognition molecule that specifically detect peptidoglycan from Gram-negative bacteria (19, 20).

Because probiotics such as *L. casei* represent a subclass of commensals that modulate mucosal innate responses and possibly exhibit an anti-inflammatory function (8), we aimed to analyze the effect of *L. casei* on epithelial inflammatory signaling induced by *S. flexneri*. Our results demonstrate that *L. casei* potently modulates pro-inflammatory pathways induced by *S. flexneri*, and by using global microarray analyses, we identify the ubiquitin/proteasome complex as the main target of *L. casei* action. Together, this study allows for a better understanding of how the commensal microflora contributes to the homeostasis of the host intestinal tract.

Materials and Methods

Cells lines, bacterial strains, and infections

Caco-2 and HEK293T epithelial cells lines were cultured with DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% nonessential amino acids (Invitrogen Life Technologies).

Invasive colonies of the *S. flexneri* serotype 5a strain M90T were isolated on Congo red agar plates (Congo red-positive colonies). One colony was then grown overnight in 7 ml of Trypticase soy broth (Difco) medium at 37°C under rotation. After dilution in fresh Trypticase soy broth, a subculture was then performed for 2 h to bring the culture to the exponential phase (OD, 600 nm; O.4). The strain SC301, a derivative of M90T after its transformation with plasmid pIL22 (encoding the afimbrial adhesin AfaE) from uropathogenic *Escherichia coli*, was used in this study (21).

The probiotic strain *L. casei* DN-114 001 was provided by Danone Vitapole. A colony of *L. casei* isolated on a Mann-Rogosa-Sharpe (MRS) agar plate was picked and cultured overnight at 37°C in 10 ml of MRS broth without agitation in absence of oxygen.

To obtain a polarized monolayer of IECs, Caco-2 cells were grown to confluency and then for 2 additional weeks to reach full differentiation and polarization. The culture medium was changed every 2 d. For the infection step, Caco-2 cells were incubated overnight without serum, with *L. casei* at a multiplicity of infection (MOI) of 100, before the addition of strain M90T-AfaE at the same MOI for 4 h. For the time-course experiment, nonpolarized Caco-2 cells were incubated at a MOI of 100 with *L. casei* for 2, 6, or 24 h. Biological duplicates were done for this time-course experiment. After washing Caco-2 cells with cold PBS, RNAs were extracted with the RNEasy mini kit (Qiagen). The quantity and quality of RNA preparations were determined, respectively, by absorbance lecture and electrophoresis using the Agilent nanochip technology.

To detect and quantify cell invasion, overnight culture of HEK cells seeded on coverslips, in the presence or absence of *L. casei*, were infected with M90T-AfaE as described above. After 1 h of infection, cells were washed in PBS and fixed with 4% paraformaldehyde. To identify intracellular vs extracellular *Shigella*, after saturation with BSA (1% in PBS), a first staining was made in the absence of permeabilization with a rabbit polyclonal Ab against *S. flexneri* LPS, followed by the addition, after washing, of a rhodamine-labeled goat Ab against rabbit Ig. The cells were then permeabilized with 0.1% Triton X-100. The same incubation with the Ab was performed, with a FITC-labeled goat Ab. Once labeled intracellular bacteria appeared green-labeled, whereas twice-labeled extracellular bacteria appeared yellow-labeled. Fluorescence detection was done using an Olympus BX50 microscope (at a ×400 magnification) and a Leica DC350F camera. Pictures were analyzed using the Leica IM50 software (version 1.2; (Leica Microsystems). Determination of the green and red pixels were performed using Photoshop 7.0 software.

Macroarrays hybridization and analysis

The PCR products of 1050 human genes were spotted in duplicate on positively charged nylon membranes as described previously (22). The macroarray design can be found on the ArrayExpress web site (www.ebi.ac.uk/arrayexpress) with the accession number A-MEXP-141. cDNA labeling and hybridization scanning were also as described previously (22).

After recording of the signals for each gene with ArrayVision and quality control of hybridizations using the luciferase signal intensity, data corresponding to all of the membranes were transformed in a $\log_2$ scale and normalized by a method derived from the variance analysis (ANOVA) to give to all membranes an equal mean signal. This statistical method estimates the weight and significance of variability sources on experimental data. For each condition, eight biological replicates were performed and hybridized onto macroarrays in which PCR products were spotted in duplicate; 16 signals for one gene were used for one experimental condition. Comparative analyses between baseline (noninfected cells) and experiment (infected cells) were done with the dChip software (23), using an unpaired Welch *t* test with a *p*-value threshold of 0.05. This software was also used for hierarchical clustering using Euclidian distance and average as a linkage method. Before clustering, the expression values for one gene across all samples are standardized to have a mean of zero. Increased or decreased values are then ranged compared with this mean. In the clustering picture, each row represents a gene and each column represents a sample. For the color scale, blue and red represent, respectively, low and high signal expression values.

GeneChip hybridization and analysis

Starting from 5 μg of total RNA, the first strand of cDNA was synthetized using T7-(dT24) oligonucleotides and Superscript II reverse transcriptase (Invitrogen Life Technologies) for 1 h at 37°C. The double-strand cDNA was then synthetized at 16°C for 2 h by the addition of DNA polymerase, DNA ligase, and RNaseH. A terminal step by T7 DNA polymerase was performed for 5 min. The resulting DNA was used to synthetize biotin-labeled cRNA via an in vitro transcription labeling kit (Enzo Diagnostics). Ten micrograms of fragmented cRNA were hybridized with the GeneChip U133A for 16 h at 60 rpm in a 45°C hybridization oven. The chips were washed and stained with streptavidin PE (Molecular Probes) in the Affymetrix fluidics station. To amplify staining, an anti-streptavidin biotinylated Ab was introduced between two streptavidin PE staining steps. After hybridization, staining, and scanning (Agilent), the chip images were analyzed for quality control before signal analysis. The chips were then normalized using the scaling factor method (with a target intensity of 100). The analysis was performed using the statistical algorithms from the MAS-5 software (Affymetrix) to obtain absolute signals, detection calls, and the associated *p* values. Transcripts that were absent both in control and experimental conditions were removed from further analysis. Comparative analysis, between control and experimental conditions, which give a signal log ratio, a change call, and an associate *p* value, was performed with MAS-5 software using the *t* test and Mann-Whitney *U* test. Because the experiments were done in duplicate, to choose the modulated genes, we selected the probe set, the change call (increase or decrease) of which appeared to be four times (each experimental point vs each control point). Hierarchical clustering was performed using dChip software with the same parameters than those described above.

PCR analysis

cDNAs were synthetized from a template of 5 μg of total RNA using oligo(dT) primers (Promega) and Superscript II (Invitrogen Life Technologies) for 1 h at 42°C. PCR was performed using 5 μl of a 1/20 dilution of the cDNA as a template in a final volume of 50 μl. The standard program used was as follows: denaturation for 5 min at 94°C, 35 cycles of 45 s treatment at 94°C, 45 s at 55°C, and 90 s at 72°C, followed by a final elongation for 7 min at 72°C. One unit of Eurobiotaq (Eurobio) was used for one PCR. After electrophoresis in a 2% agarose gel containing ethidium bromide, PCR products were quantified using the ImageQuant software (Amersham Biosciences). The primers used are described in Table I.

NF-κB reporter gene assays and I-κBα evaluation

Experiments were conducted as reported previously (19). Briefly, 5×10^5 HEK293 cells were transiently transfected using 75 ng of the NF-κB luciferase reporter system, and NF-κB-dependent luciferase activity was measured 20 h after transfection. NF-κB-dependent luciferase assays were performed in duplicate, and data represent at least three independent experiments. Data show mean ± SE.

For experiments in which *L. casei* was added before stimulation with either *S. flexneri* or TNF-α, the following procedure was set up: HEK293 cells were seeded at 5×10^5 cells/ml and either left unstimulated (for Western blotting experiments) or transfected with 75 ng of the NF-κB luciferase reporter gene (for NF-κB-dependent luciferase assays) as described above. Twenty hours later, the incubation medium was changed and replaced by fresh medium containing either MRS medium alone or *L. casei* grown in MRS buffer (5×10^7 bacteria/ml). After an overnight incubation, cells were rinsed three times with PBS, and fresh medium was

Table I. *PCR primers used for PCRs*

Gene Name	Forward	Reverse	Length
GAPDH	TGAAGGTCGGAGTCAACGGATTTGGT	CATGTGGGCCATGAGGTCCACCAC	983
Amphiregulin	CTAGTAGTGAACCGTCCTCG	CTCCTTCATATTTCCTGACG	489
CXCL1	TGTCAACCCCAAGTTAGTTC	TCAATAATTAAGCCCCTTTG	400
CXCL2	CCAAAGTGTGAAGGTGAAGT	ATGGGAGAGTGTGCAAGTAG	400
IL-8	ATGACTTCCAAGCTGGCCGTGGCT	TCTCAGCCCTCTTCAAAAACTTCTC	289
ICAM-1	AGTCACCTATGGCAACGACTCC	GGCCATACAGGACACGAAGCT	401
Rbx-1	AAGAAGCGCTTTGAAGTGAA	GGTAACAGCAGGGAAAGTCA	339
Skp-1	GGAAATTGCCAAACAATCTG	TTGAAGGTCTTGCGAATCTC	366
Proteasome ATPase 1	CAATCATGCCATCGTGTCTA	GAGCCAACCACTCTCAAGAA	405
Proteasome ATPase 6	CAGCTGGACTGCAATTTCTT	GCGAACATACCTGCTTCAGT	494

added. HEK293 cells were then left unstimulated, stimulated with TNF-α (100 ng/ml), or infected with *S. flexneri* M90T-AfaE (5×10^7 bacteria/ml) for variable periods (4 h in the case of NF-κB luciferase reporter assays), before lysis of the cells.

Western blot analysis

After overnight incubation of Caco-2 cells in the presence or absence of *L. casei*, the cells were stimulated either with M90T-AfaE or with TNF before lysis by the addition of 200 μl of Laemmli solution. After heating 5 min at 90°C, 10 μl of lysate were loaded in a 10% acrylamide SDS-PAGE. After migration, proteins were transferred onto nitrocellulose by semidry transfer. After blocking by PBS/5% milk, the membrane was incubated overnight with anti-I-κBα, antiphosphorylated I-κBα, anti-Rbx1, or anti-polyubiquitin Abs (all purchased from Santa Cruz Biotechnology) or with anti-β-tubulin (Sigma-Aldrich) at 1/500 in PBS/milk. After three washes in PBS/Tween, the membrane was incubated with a peroxydase-labeled secondary Ab (1/1000) for 1 h. After washing, the membrane was incubated for 5 min with ECL$^+$ chemiluminescence reagent (Amersham Biosciences). Kodak film was then exposed for different time periods to the membrane.

Results

Gene expression patterns in Caco-2 cells during infection with Shigella

Until recently, GeneChip studies have been hampered by some technical concerns, including the absence of key genes and redundancy. In an effort to characterize with precision the global

Table II. *Macroarray analysis of polarized Caco-2 cell gene expression during* Shigella *infection*[a]

Probe Set	NI (mean ± SD)	M90T (mean ± SD)	P value	Fold Change
Vimentin	14.62 ± 0.18	14.15 ± 0.13	0.041027	−1.39
Postmeiotic segregation 1	14.32 ± 0.09	13.97 ± 0.09	0.008549	−1.27
Human chemokine α 3 (CKA-3)	13.44 ± 0.1	13.1 ± 0.09	0.015804	−1.27
Collagen, type II, α 1	14.23 ± 0.12	13.91 ± 0.09	0.038098	−1.25
PAC 179D3	13.8 ± 0.08	13.55 ± 0.05	0.009966	−1.20
Uk-13	13.59 ± 0.05	13.39 ± 0.06	0.015796	−1.16
v-erb-a avian erythroblastic leukemia viral oncogene	13.35 ± 0.09	13.14 ± 0.03	0.045152	−1.16
Bullous pemphigoid antigen 1	13.25 ± 0.06	13.05 ± 0.05	0.014533	−1.15
FK506-binding protein 1A (12 kDa)	13.75 ± 0.07	13.55 ± 0.06	0.043947	−1.15
Flavin containing monooxygenase 4	13.11 ± 0.03	12.95 ± 0.04	0.004456	−1.13
FK506-binding protein 4 (59 kDa)	13.36 ± 0.06	13.19 ± 0.04	0.031667	−1.12
Bcl-2 binding protein	13.35 ± 0.05	13.2 ± 0.03	0.012662	−1.11
Heparan sulfate proteoglycan 2	13.15 ± 0.05	13.02 ± 0.05	0.041943	−1.10
IL-2	13.09 ± 0.09	13.32 ± 0.04	0.023695	1.18
CXCL1	13.09 ± 0.08	13.33 ± 0.08	0.042154	1.19
IEX	13.21 ± 0.05	13.45 ± 0.08	0.016412	1.19
Connective tissue growth factor	13.05 ± 0.05	13.36 ± 0.07	0.000867	1.24
CXCL3	12.9 ± 0.07	13.22 ± 0.12	0.037018	1.24
Coproporphyrinogen oxidase	14.19 ± 0.08	14.53 ± 0.1	0.01293	1.27
SOCS-3	13.34 ± 0.12	13.68 ± 0.1	0.032277	1.27
CL100 mRNA for protein tyrosine phosphatase	12.9 ± 0.07	13.24 ± 0.08	0.003215	1.27
IRF-1	12.99 ± 0.06	13.37 ± 0.14	0.019869	1.30
Ribosomal protein S3	17.22 ± 0.11	17.63 ± 0.09	0.009622	1.32
Ryudocan	13.99 ± 0.07	14.4 ± 0.14	0.02105	1.32
MAD-3 (IK-B like activity)	12.95 ± 0.07	13.37 ± 0.13	0.007291	1.34
CCL20 (MIP-3 α)	13.61 ± 0.12	14.1 ± 0.18	0.032425	1.39
GRO-2 oncogene	12.91 ± 0.1	13.42 ± 0.18	0.018873	1.42
Heat shock 70 KDa protein 6	14.09 ± 0.12	14.61 ± 0.12	0.005595	1.42
Homo sapiens chemokine exodus-1	12.73 ± 0.1	13.27 ± 0.22	0.03742	1.45
TNF ip20	13.16 ± 0.03	13.74 ± 0.14	0.000984	1.49
Amphiregulin	13.92 ± 0.16	14.52 ± 0.1	0.003459	1.52
CXCL2	13.37 ± 0.06	14.01 ± 0.23	0.015317	1.56
EST-2	14.46 ± 0.2	15.11 ± 0.14	0.011017	1.58
v-jun	13.4 ± 0.12	14.14 ± 0.13	0.000343	1.67
Superoxide dismutase 2, mitochondrial	16.83 ± 0.25	17.66 ± 0.3	0.041135	1.77
Heat shock 70 KDa protein 1A	15.12 ± 0.18	16.28 ± 0.12	0.000013	2.23

[a] Signal intensity resulting from macroarray hybridizations done with eight biological replicates of M90T infected Caco-2 cells were normalized and log2 transformed. Data analysis using the unpaired Welch *t* test from the dChip software gives a fold change between experimental point and baseline in a linear scale and an associated *p* value. Results with a $p < 0.05$ were considered as statistically significant.

response of cells infected by a bacterial pathogen, we have developed a macroarray-based procedure in which PCR products of 1050 genes of interest, including cytokines, chemokines, transcription factors, etc. (22), have been spotted. Using this tool, we aimed to identify the repertoire of genes modulated after infection of polarized Caco-2 cells with the invasive *S. flexneri* strain M90T-AfaE. Radioactive cDNA was hybridized onto macroarray nylon membranes, and after normalization plus comparative analysis using dChip, 36 genes modulated during infection with M90T with statistical significance ($p < 0.05$) were identified (Table II). Interestingly, genes encoding chemoattractant chemokines with pro-inflammatory functions such as CXCL1–3 and CCL20 were found up-regulated after *Shigella* infection. Importantly, results obtained by macroarray analyses confirm and further expand results obtained during *Shigella* infection of nonconfluent Caco-2 cells using Affimetrix technology (24).

Modulation of the response of Caco-2 cells infected with Shigella *by* L. casei

To observe the effect of *L. casei* DN-114 001 on the inflammatory process occurring during *Shigella* infection of IECs, polarized Caco-2 cells were preincubated overnight with *L. casei* before infection with M90T-AfaE. After RNA extraction, hybridizations to the macroarray membranes were performed. Interestingly, hierarchical clustering identified *L. casei*-mediated modulation of *Shigella*-induced responses of epithelial cells (Fig. 1). Among these, some key pro-inflammatory genes were found to be down-regulated when Caco-2 cells were preincubated with the probiotic bacteria before infection with *Shigella*. This was the case for the expression of CXCL1 and CXCL2, two chemokines involved in chemoattraction of polymorphonuclear cells, and for the expression of the dendritic cell chemoattractant CCL20.

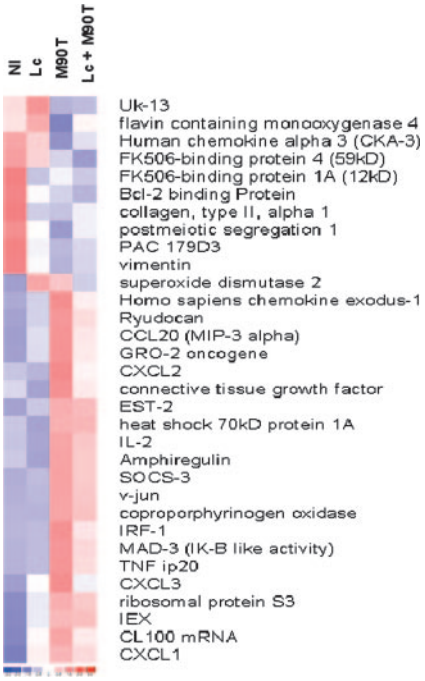


FIGURE 1. Hierarchical clustering modulated genes during infection of Caco-2 cells with *Shigella*, in the presence or absence of *L. casei*. Caco-2 cells were incubated overnight in the presence or absence of *L. casei* and challenged for 3 h with M90T-AfaE, the virulent strain of *S. flexneri*. Radioactive nucleotides were incorporated during cDNA synthesis. The labeled probes were hybridized onto macroarrays consisting of 1050 human genes represented by PCR products spotted onto nylon membranes. After washings and scanning, signal detection and normalization were performed allowing group comparisons. Modulated genes were then clustered using dChip software. Each row represents a gene, and each column represents the mean expression for each replicate. The red color represents an expression level above the mean expression of a gene across all samples, and the blue color represents an expression level lower than the mean. For each condition, eight biological replicates were made.

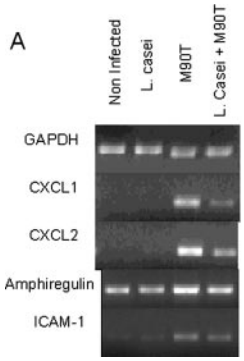
When macroarray-based technologies are used, it is crucial to further confirm the results obtained by individual RT-PCR. Using this approach, we could validate that expression of CXCL1 and CXCL2 was down-regulated (both by ~50%) in conditions of coinfection with *L. casei* and M90T-AfaE compared with M90T-AfaE alone (Fig. 2). Moreover, in a previous study (24), we demonstrated that the expressions of ICAM-1 and amphiregulin were up-regulated during *Shigella* infection. In this study, we report that preincubation of Caco-2 cells with *L. casei* prevented increased transcription of these genes after infection with *Shigella* (Fig. 2).

The above results pointed to a role of *L. casei* in the specific modulation of a selected repertoire of *Shigella*-induced genes. However, to ascertain that these observations were not an indirect consequence of the experimental set-up used, we aimed to investigate whether coculture of Caco-2 cells with *L. casei* interferes with *Shigella* invasion. Using double-fluorescence labeling, followed by counting of intracellular vs extracellular *Shigella*, we observed that regardless of the absence or presence of *L. casei* in the culture medium, the ratio of intracellular vs extracellular *Shigella* remained unchanged (1.91 ± 0.74 or 1.70 ± 0.66, respectively).

Together, these results demonstrate that *L. casei* selectively down-regulates the expression of some key factors induced after infection of IECs with *Shigella*.

L. casei *blocks NF-κB activation induced by* S. flexneri

Because a number of the *Shigella*-induced genes identified above are under the control of the NF-κB transcription complex, we aimed to investigate whether *L. casei* acted directly on this signaling pathway. Strikingly, using a NF-κB-dependent luciferase reporter gene assay, we observed that overnight preincubation of HEK293 cells with *L. casei* repressed NF-κB activation induced by *Shigella* (Fig. 3). We then investigated the nature of this blockage at the molecular level. In epithelial cells, the signaling pathway leading to the activation of NF-κB after *S. flexneri* infection was recently identified. It involves the intracellular peptidoglycan-recognition molecule Nod1 and a signaling cascade involving Rip2/RICK/Cardiak, the IKK complex, and the inhibitory protein I-κBα



B

	Non Infected	L. casei	M90T	L. Casei + M90T
CXCL1	2.3	12.3	133	41.7
CXCL2	3.6	5.2	143	51.9
Amphiregulin	134	179	243	190
ICAM-1	92.5	106	221	143

FIGURE 2. Anti-inflammatory effect of *L. casei*. The cells were treated as described in *Material and Methods* and in the legend to Fig. 1. After RNA extraction, cDNA was synthetized using Oligo-dT and reverse transcriptase enzyme. Five microliters of a 1/20 dilution were used as a template for the PCR. *A*, Confluent, polarized Caco-2 cells were used. *B*, Densitometric quantification of PCR products ((gene/GAPDH) × 100).

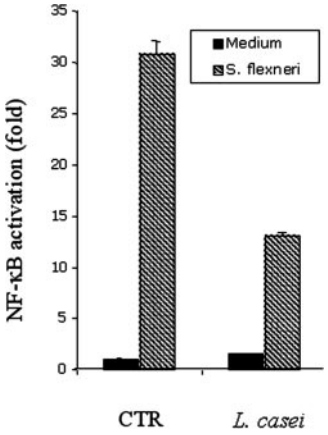


FIGURE 3. Inhibition of *Shigella*-induced NF-κB activation by *L. casei*. Overnight preincubation of HEK293 cells with *L. casei* blocks NF-κB activation induced by the invasive bacteria *S. flexneri*. CTR, Control.

(25). We analyzed whether preincubation with *L. casei* could modulate the expression of selected members of this signaling cascade. Although the expressions of Rip2 and IKKα were not modified by pretreatment with *L. casei* (Fig. 4*A*), we noticed a substantial increase of I-κBα expression after overnight coculture of HEK293 cells with *L. casei* (Fig. 4*A*).

Infection of epithelial cells with *S. flexneri* induces rapid and dramatic degradation of I-κBα, resulting in the release of a free NF-κB complex that translocates to the nucleus to induce transcription of NF-κB-dependent pro-inflammatory genes. Indeed, we could confirm these observations in a kinetic study of I-κBα degradation ranging from 10 to 40 min after infection (Fig. 4*B*, *top panel*). In similar conditions, overnight preincubation of HEK293

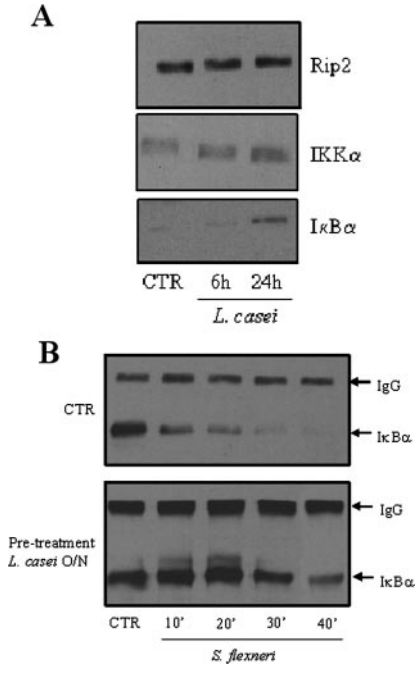


FIGURE 4. *L. casei* infection results in up-regulation of I-κBα protein and inhibits *Shigella*-induced I-κBα degradation. *A*, Overnight preincubation of HEK293 cells with *L. casei* induces the up-regulation of I-κBα protein, while leaving other proteins of the Nod1-dependent signaling pathway unchanged. *B*, Overnight (O/N) preincubation of HEK293 cells with *L. casei* blocks I-κBα degradation induced by the invasive bacteria *S. flexneri*. CTR, Control.

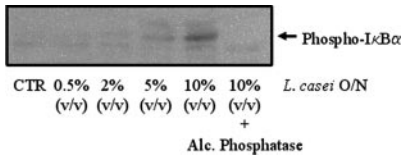


FIGURE 5. *L. casei* infection results in up-regulation of the phosphorylated form of the I-κBα protein. Overnight (O/N) preincubation of HEK293 cells with *L. casei* (ranging from 2.5 10^6 to 5.10^7 bacteria/ml) induced a dose-dependent increase of I-κBα protein phosphorylation as determined by Western blotting using a specific polyclonal anti-human phospho-I-κBα Ab. CTR, Control.

cells with *L. casei* resulted in a strong inhibition of *S. flexneri*-induced I-κBα degradation. Instead, degradation of I-κBα was observed only at later time points and remained partial (Fig. 4*B*, *bottom panel*). Interestingly, a slower migrating band likely corresponding to a phosphorylated form of I-κBα was transiently detected, but only in cells preincubated with *L. casei*. I-κBα degradation is mediated by the ubiquitin-proteasome system and the phosphorylation of I-κBα is a signal that targets the protein to degradation. In unstimulated cells, phospho-I-κ Bα is hardly detectable because the protein is immediately targeted for degradation. Because in epithelial cells incubated with *L casei* we could detect phospho-I-κBα (Fig. 5), we anticipated that the effect of *L. casei* on I-κBα may be related to altered degradation of the protein by the ubiquitin-proteasome system.

These results suggested that the anti-inflammatory effect of *L. casei* is largely mediated through specific modulation of the I-κBα protein level. However, I-κBα lays relatively downstream in the NF-κB signaling cascade, and several pro-inflammatory stimuli lead to its degradation. We therefore investigated whether *L. casei* could down-regulate NF-κB activation induced by TNF-α, another well characterized pro-inflammatory stimulus. Indeed, we could observe that overnight preincubation of HEK293 cells with *L. casei* potently repressed NF-κB activation induced by TNF-α (Fig. 6*A*). Moreover, a kinetic study of I-κBα degradation ranging from

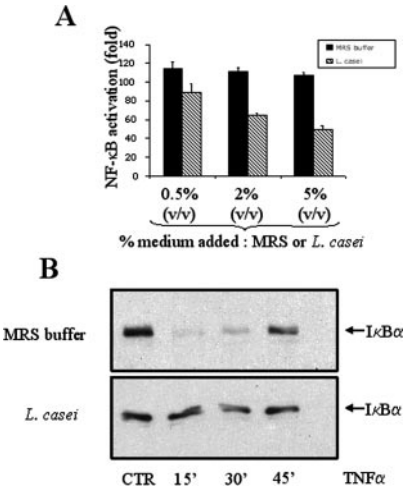


FIGURE 6. Inhibition of TNF-α-induced NF-κB activation by *L. casei*. *A*, HEK293 cells were preincubated overnight with *L. casei* or MRS buffer (control medium) before stimulation for 4 h with TNF-α. Three bacterial concentrations were used: 5.10^6, 2.10^7, or 5.10^7 bacteria/ml. *B*, HEK293 cells were preincubated overnight with *L. casei* or MRS buffer (control medium) before stimulation for various times (15, 30, or 45 min) with TNF-α, followed by lysis of the cells. The I-κBα protein content was determined by Western blotting using a polyclonal anti-human I-κBα Ab. CTR, Control.

Table III. *Determination of modulated probe sets during time course culture of Caco-2 cells with L. casei*[a]

Time Course	Probe Set Number	
	Decrease	Increase
2 h	53	35
6 h	63	59
24 h	357	237

[a] Caco-2 cells were cultured 2, 6, or 24 h with *L. casei.* After RNA extraction, labeling and hybridizations onto U133A Affymetrix GeneChip were performed. The number represents the modulated probe set with a fold change > 2.

15 to 45 min after stimulation with TNF-α showed that I-κBα degradation was strongly decreased if HEK293 cells had been pre-incubated overnight with *L. casei* (Fig. 6*B*). Together, these results strongly suggest that the anti-inflammatory properties of *L. casei* largely depend on the modulation of I-κBα expression and/or stability.

Global transcriptomic analysis of epithelial cells incubated with L. casei

The above results demonstrate a role for *L. casei* in the specific modulation of NF-κB-driven genes induced after *Shigella* infection. However, it remains possible that other key signaling pathways are targets of *L. casei*-induced modulatory properties. To gain more insight into the global effects mediated by *L. casei* on host cell signaling, we performed a large-scale gene expression study (14,500 well characterized human genes; Affymetrix Genechips U133A) on Caco-2 cells cocultured with the probiotic strain for 2, 6, or 24 h. The entire set of results is available online (supplemental Table S1)[5], and Table III represents the number of probe sets modulated during Caco-2 cell culture in the presence of *L. casei*. Interestingly, most of the modulation was observed after 24 h of incubation. Because all of the results are available online, and to avoid to present an extensive list of modulated genes, we have focused in this study on four functional clusters (defined using NettAffyx, the database from Affymetrix) that appeared to be modulated most extensively by *L. casei* (Table IV and see below).

Group 1: genes involved in control of the cell cycle. *L. casei* induced both positive and negative modulation of expression of genes involved in the regulation of the cell cycle. Indeed, tumor suppressor genes, such as tumor rejection Ag (gp96) and sel-1 suppressor of lin-12, were up-regulated during incubation with *L. casei*, whereas the expression of the gene encoding prohibitin, a negative regulator of cell proliferation, was down-regulated.

Group 2: genes involved in the control of apoptosis. Expression of two genes encoding proteins involved in the inhibition of apoptosis (Fas apoptotic inhibitory molecule and cytokine-induced apoptosis inhibitor) were down-regulated, and expression of BCL2/adenovirus E1B was increased. It should be noted that the expression of Nalp2, encoding a part of the inflammasome (26), decreased during the incubation of Caco-2 cells with *L. casei*, suggesting its potential involvement in the anti-inflammatory properties of the probiotic bacteria. In addition, the expression of programmed cell death 4, a tumor suppressor (27), was positively modulated.

Group 3: genes involved in hypoxia. Expression of two genes under the control of the hypoxia-inducible factor-1 (HIF-1) transcription factor (*hypoxia up-regulated 1* and *HIF-1 response RTP801*) was induced during the incubation of Caco-2 cells with *L. casei*.

Group 4: genes belonging to the ubiquitination/degradation pathway. Twenty-five genes involved in the protein ubiquitination and/or degradation process were found to be positively or negatively modulated during incubation of Caco-2 cells with *L. casei*. The transcription of genes encoding three ubiquitin-conjugating enzymes, E2D, E2L, and E2N, and four subunits of the 26S proteasome were down-regulated. Another gene, the expression of which decreased during this incubation, was *rbx*-1/*roc*-1/*hrt*-1, which encodes a protein belonging to the E3 ligase complex. We confirmed by individual PCR on a set of these selected genes their down-regulated expression after treatment with *L. casei* (Fig. 7*A*). It is striking to note that, among all of the genes modulated by *L. casei*, the group of genes implicated in ubiquitination/degradation (group 4) is, by far, the most represented. This observation suggests that *L. casei* is capable of establishing a program leading to the down-regulation of the ubiquitin-mediated degradation of proteins. Moreover, because the ubiquitin system is responsible for the degradation of I-κBα, this observation strongly supports our conclusions that modulating the stability of I-κBα represents a major target of *L. casei*.

Among the 25 genes of the ubiquitination/degradation pathway that were identified, we noticed that Rbx-1 expression was down-regulated to a greater extent (~4-fold decrease) by *L. casei* than the others (Table IV). This prompted us to investigate whether the effects of *L. casei* on the NF-κB pathway could be accounted for by the specific down-regulation of Rbx-1. First, the expression of Rbx-1 at the protein level was monitored in *L. casei*-treated cells and was found to be reduced by ~50% (Fig. 7*B*). Second, overexpression of exogenous epitope-tagged Rbx-1 in *L. casei*-treated cells was performed, and TNF-mediated (as well as *Shigella*-mediated) degradation of I-κBα was monitored (data not shown). However, restoring Rbx-1 expression was found to be insufficient to recapitulate the effects of *L. casei* on the ubiquitin/degradation pathway. As a consequence, it is likely that *L. casei* down-regulates this cellular process via the targeting of multiple effectors. Accordingly, we observed that the global pattern of ubiquitinated proteins after *L. casei* treatment closely resembles the one of cells treated with MG132, a broad inhibitor of multiple ubiquitin/proteasome-dependent pathways (Fig. 7*C*). Together, this large-scale analysis of *L. casei*-modulated genes in Caco-2 cells strongly supports the conclusion that this probiotic bacteria displays anti-inflammatory properties through the targeting of the ubiquitin/proteasome system.

Discussion

In this study, we have investigated the effect of coculture of IECs with the probiotic bacteria *L. casei*. We were interested in two questions of fundamental importance: 1) Does this probiotic bacteria display protective properties on IECs infected with a pathogenic entero-invasive bacteria? and 2) What is the pattern of transcriptional responses of the epithelial cells cocultured for various periods with *L. casei*? To gain insight into these questions, we embarked on a project to identify the global repertoire of host responses to *L. casei* using, for the first time on this topic, the technologies of macroarray analysis (1,050 genes selected) associated with DNA chip hybridization (Affymetrix; 14,500 genes) and biochemical analysis. Our results clearly identify a role for *L. casei* in the specific modulation of a repertoire of genes associated with the ubiquitin/proteasome pathway. Importantly, the down-regulation of this set of ubiquitin system-associated genes appears to play a crucial role in the modulation of pro-inflammatory pathways in IECs. Indeed, when pro-inflammatory stimuli, such as

[5] The online version of this article contains supplemental material.

Table IV. *Gene expression modulation during L. casei time course*[a]

GO Biological Process[b]	Probe Set	RefSeq ID	Gene Name	Modulation[c]	Fold Change[d]
Group 1: Cell growth/cell cycle	201278_at	NM_001343	Disabled homolog 2, mitogen-responsive phosphoprotein (*Drosophila*)	D	−4.68
	201202_at	NM_002592	Proliferating cell nuclear antigen	D	−4.29
	216237_s_at	NM_006739	MCM5 minichromosome maintenance deficient 5, cell division cycle 46	D	−4.07
	203625_x_at	NM_005983	S-phase kinase-associated protein 2 (p45)	D	−3.93
	204318_s_at	NM_016426	G-2 and S-phase expressed 1	D	−3.42
	204709_s_at	NM_004856	Kinesin family member 23	D	−3.14
	200659_s_at	NM_002634	Prohibitin	D	−3.08
	209662_at	NM_004365	Centrin, EF-hand protein, 3 (CDC3 1 homolog, yeast)	D	−2.98
	204162_at	NM_006101	Kinetochore associated 2	D	−2.88
	214710_s_at	NM_031966	Cyclin B1	D	−2.83
	207165_at	NM_012484	Hyaluronan-mediated motility receptor (RHAMM)	D	−2.73
	202883_s_at	NM_002716	Protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), β	D	−2.73
	208712_at	NM_001758	Cyclin D1 (PRAD1: parathyroid adenomatosis 1)	D	−2.51
	213222_at	NM_015192	Phospholipase C, β 1 (phosphoinositide-specific)	D	−2.51
	203145_at	NM_006461	Sperm associated antigen 5	D	−2.46
	204444_at	NM_004523	Kinesin family member 11	D	−2.46
	202240_at	NM_005030	Polo-like kinase 1 (*Drosophila*)	D	−2.42
	200959_at	NM_004960	fusion (involved in t(12;16) in malignant liposarcoma)	D	−2.38
	218350_s_at	NM_015895	geminin, DNA replication inhibitor	D	−2.34
	203078_at	NM_003591	Cullin 2	D	−2.30
	203362_s_at	NM_002358	MAD2 mitotic arrest deficient-like 1 (yeast)	D	−2.22
	212426_s_at	NM_006826	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation	D	−2.22
	212949_at	NM_015341	Barren homolog (*Drosophila*)	D	−2.18
	208727_s_at	NM_001791	cell division cycle 42 (GTP binding protein, 25 kDa)	D	−2.18
	201938_at	NM_004642	CDK2-associated protein 1	D	−2.14
	203740_at	NM_005792	M-phase phosphoprotein 6	D	−2.03
	201664_at	NM_0012799	SMC4 structural maintenance of chromosomes 4-like 1 (yeast)	D	−1.90
	218009_s_at	NM_003981	Protein regulator of cytokinesis 1	D	−1.90
	201725_at	NM_006023	Chromosome 10 open reading frame 7	D	−1.87
	220789_s_at	NM_004749	Transforming growth factor β regulator 4	D	−1.87
	204817_at	NM_012291	Extra spindle poles like 1 (*S. cerevisiae*)	D	−1.87
	201186_at	NM_002337	Low-density lipoprotein receptor-related protein associated protein 1	D	−1.65
	221509_at	NM_003677	Density-regulated protein	D	−1.57
	201173_x_at	NM_006600	Nuclear distribution gene C homolog (*A. nidulans*)	D	−1.46
	217839_at	NM_006070	TRK-fused gene	I	1.87
	219910_at	NM_007076	Huntingtin interacting protein E	I	2.38
	203226_s_at	NM_005981	Sarcoma amplified sequence	I	2.55
	202205_at	NM_003370	Vasodilator-stimulated phosphoprotein	I	3.86
	202061_s_at	NM_005065	sel-1 suppressor of lin-12-like (*C. elegans*)	I	3.86
	210513_s_at	NM_003376	Vascular endothelial growth factor	I	4.07
	205569_at	NM_014398	Lysosomal-associated membrane protein 3	I	4.92
	200598_s_at	NM_003299	Tumor rejection antigen (gp96) 1	I	5.46
Group 2: Apoptosis	220643_s_at	NM_018147	Fas apoptotic inhibitory molecule	D	−5.10
	221690_s_at	NM_017852	NACHT, leucine rich repeat and PYD containing 2 (Nalp2)	D	−4.68
	202268_s_at	NM_003905	Amyloid β precursor protein binding protein 1, 59 KDa	D	−2.14
	220044_x_at	NM_006107	Cisplatin resistance-associated overexpressed protein	D	−2.14
	208424_s_at	NM_020313	Cytokine induced apoptosis inhibitor 1	D	−2.11
	219275_at	NM_004708	Programmed cell death 5	D	−1.65
	203489_at	NM_006427	CD27-binding (Siva) protein	D	−1.49
	221479_s_at	NM_004331	BCL2/adenovirus E1B 19 kDa interacting protein 3-like	I	2.30
	202731_at	NM_014456	Programmed cell death 4 (neoplastic transformation inhibitor)	I	3.36
	202014_at	NM_014330	Protein phosphatase 1, regulatory (inhibitor) subunit 15A	I	7.21
Group 3: Hypoxia	200825_s_at	NM_006389	Hypoxia up-regulated 1	I	3.86
	202887_s_at	NM_019058	HIF-1 response RTP801	I	4.92
Group 4: Ubiquitination/degradation	218117_at	NM_014248	Ring-box 1	D	−4.07
	212751_at	NM_003348	Ubiquitin-conjugating enzyme E2N (UBC13 homolog, yeast)	D	−2.88
	201377_at	NM_014847	Ubiquitin associated protein 2-like	D	−2.42
	201498_at	NM_003470	Ubiquitin specific protease 7 (herpes virus-associated)	D	−2.34
	211764_s_at	NM_003338	Ubiquitin-conjugating enzyme E2D 1 (UBC4/5 homolog, yeast)	D	−2.22

Table IV. *Continued*

GO Biological Process[b]	Probe Set	RefSeq ID	Gene Name	Modulation[c]	Fold Change[d]
	201199_s_at	NM_002807	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 1	D	−2.00
	204219_s_at	NM_002802	Proteasome (prosome, macropain) 26S subunit, ATPase, 1	D	−1.97
	200683_s_at	NM_003347	Ubiquitin-conjugating enzyme E2L 3	D	−1.93
	200988_s_at	NM_005789	Proteasome (prosome, macropain) activator subunit 3 (PA28 γ Ki)	D	−1.87
	201699_at	NM_002806	Proteasome (prosome, macropain) 26S subunit, ATPase, 6	D	−1.83
	202151_s_at	NM_016172	Ubiquitin associated domain containing 1	D	−1.77
	212987_at	NM_012347	F-box protein 9	D	−1.71
	202038_at	NM_004788	Ubiquitination factor E4A (UFD2 homolog, yeast)	D	−1.65
	202128_at	NM_014821	KIAA0317	D	−1.62
	200786_at	NM_002799	Proteasome (prosome, macropain) subunit, β type, 7	D	−1.57
	201671_x_at	NM_005151	Ubiquitin specific protease 14 (tRNA-guanine transglycosylase)	D	−1.46
	212576_at	NM_015246	Mahogunin, ring finger 1	D	−1.37
	208723_at	NM_004651	Ubiquitin specific protease 11	D	−1.21
	218582_at	NM_017824	Ring finger protein 153	I	1.74
	201133_s_at	NM_014819	Praja 2, RING-H2 motif containing	I	1.97
	208980_s_at	NM_021009	Ubiquitin C	I	2.07
	201881_s_at	NM_005744	Ariadne homolog, ubiquitin-conjugating enzyme E2 binding protein, 1	I	2.14
	221962_s_at	NM_003344	Ubiquitin-conjugating enzyme E2H (UBC8 homolog, yeast)	I	2.18
	208663_s_at	NM_003316	Tetratricopeptide repeat domain 3	I	2.34
	36564_at	NM_153341	BR domain containing 3	I	3.03

[a] The cells were treated as described in Table III.
[b] Four different pathways were selected using the NetAffyx database.
[c] D and I indicate, respectively, whether the gene expression decreases or increases between the experimental point and the baseline (Caco-2 cells alone).
[d] Fold change between the experimental point and baseline in a linear scale.

Shigella infection or TNF treatment, were used to stimulate these cells, we observed a key role of *L. casei* preculture in dampening these responses. Moreover, we could pinpoint the molecular basis of this modulation by the observation that *L. casei* specifically targets the stability of I-κBα, the specific NF-κB inhibitor, thereby shutting down this major pro-inflammatory pathway.

Our study confirms and also extends the hypothesis that certain commensal microorganisms have the potential to actively influence the homeostatic control of intestinal inflammation (2). Indeed, coculture of human tissues with *L. casei* significantly reduced TNF-α release from Crohn's disease patients' inflamed mucosa (8) even in presence of a nonpathogenic *E. coli* that alone enhanced TNF-α release from inflamed tissues (9). In addition, in a mouse model of skin hypersensitivity, ingestion of *L. casei* was demonstrated to reduce skin inflammation induced by dinitrofluorobenzene (28). Exposure of IECs to nonpathogenic *Salmonella* spp. could mediate anti-inflammatory signals by preventing ubiquitination of I-κBα (13). Recently, another anti-inflammatory mechanism induced by a commensal bacteria strain has been described. *B. thetaiotaomicron* attenuates pro-inflammatory cytokine expression by inducing the nuclear export of complexes formed by NF-κB and peroxisome proliferator-activated receptor-γ (29). This set of results indicates that preincubation with certain probiotic strains such as *L. casei* decreases NF-κB-driven activation of pro-inflammatory genes. In contrast, it has been shown that other commensals, such as *E. coli* MG 1655, induce NF-κB activation in IECs through the TLR5 signaling pathway (30). It must be noted that, in the present study, the two bacterial strains used, *L. casei* and *S. flexneri*, are not flagellated; thus, signal transduction via flagellin and TLR5, a major pro-inflammatory pathway described at the intestinal epithelial level (30), does not occur.

Among the genes that have been found to be modulated by *L. casei* through the Affymetrix study, we have identified a set of genes encoding proteins that control the cell cycle. Hence, the expressions of *sel-1* and *gp96*, two tumor suppressor genes, were found to be up-regulated during incubation with *L. casei*. A high level of Sel-1 has been suggested to correlate with the decrease of breast tumor growth (31), and gp96 not only elicits immune responses to tumors by inducing a $CD8^+$ T cell response (32) but also plays an adjuvant role in antitumor immune responses (33).

Another important pathway modulated by *L. casei* was shown to involve hypoxia-related genes. The level of transcription of *vegf* was found increased during incubation of Caco-2 cells with *L. casei*. This observation could prove of importance because VEGF is a growth factor that plays a key role in angiogenesis (34). Transcription of *vegf* is under the control of the transcription factor HIF-1 (35). Interestingly, two other hypoxia-responsive genes, both involved in apoptosis, were found up-regulated when Caco-2 cells were incubated with *L. casei*: the hypoxia up-regulated 1 gene (ORP150), which displays a protective function in hypoxia-conditioned cells (36), and *RTP801* gene, which inhibits hypoxia-mediated apoptosis (37). The transcription of other genes known to be under the control of HIF-1, such as erythropoietin, was not modulated during such incubation. This is likely due to the restricted transcriptional program of Caco-2 cells. Significance as well as the mechanisms involved in the establishment of hypoxia sensing by Caco-2 cells during incubation with *L. casei* remain to be further analyzed. In particular, it will be of interest to determine whether these microorganisms actively induce this process or whether cellular hypoxia is an indirect consequence of extracellular consumption of oxygen or Fe^{3+} by the bacteria.

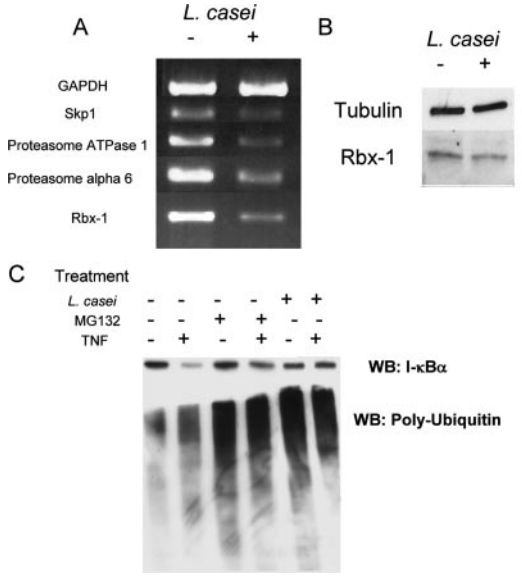


FIGURE 7. *L. casei* induced a down-regulation of genes involved in ubiquitination/degradation processes. Caco-2 cells were cultured overnight with *L. casei* with a MOI of 100. *A*, After washes with PBS and RNA extraction with the Rneasy mini kit, cDNA was synthetized using oligo-d(T) and reverse transcriptase. PCRs were performed as described in the legend to Fig. 2. *B*, After washes with PBS and lysis with Laemmli buffer, aliquots of the lysates were loaded on 15 or 10% SDS-PAGE for the subsequent detection of Rbx-1 or tubulin, respectively. The Rbx-1 or tubulin protein contents were determined by Western blotting using a polyclonal anti-human Rbx-1 or a monoclonal anti-human tubulin Ab, respectively. *C*, HEK cells were treated overnight with *L. casei* or for 6 h with 50 μM of the proteasome inhibitor MG-132 before incubation with or without TNF. The I-κBα protein content and global ubiquitinated proteins were determined by Western blotting (WB) using a polyclonal anti-human I-κBα Ab or monoclonal anti-ubiquitin Ab, respectively.

Because the expression of genes involved in different pathways (i.e., cell cycle, response to hypoxia, NF-κB-dependent transcription) was found to be modified during incubation of Caco-2 cells with *L. casei*, we investigated whether a common signaling pathway could account for these diverse effects. Of interest, these three pathways are regulated by a similar ubiquitination/proteasome system (38–41). In our time-course study using Affymetrix technology, we observed that a large set of genes involved in the ubiquitination/degradation pathways was modulated by *L. casei*. In light of our results, it appears very likely that the specific modulation of the ubiquitin/proteasome system represents a main target of action of *L. casei* on host cells and that this effect would in turn be responsible for the alteration of several signaling pathways, such as those involving the cell cycle and hypoxia.

In agreement with our findings that *L. casei* affects the ubiquitin/proteasome pathway, Caco-2 cells coincubated with *L. casei* showed stabilization of I-κBα even after subsequent stimulation by *Shigella* or TNF-α. Thus, the anti-inflammatory effects of *L. casei* are likely mediated by its effects on the ubiquitin/proteasome system and the consequent dampening of NF-κB-driven pro-inflammatory signals.

Convergent pieces of evidence suggest that, through their ability to modulate inflammatory pathways, some commensal bacteria contribute to the homeostasis of the intestinal epithelium. Our observations, and also the results from other groups, will impact on the understanding of the mechanisms responsible for some of the beneficial effects of probiotics on IBDs. This knowledge will contribute to offer, in the near future, new therapeutic means to counteract the inflammatory disorders observed in human pathologies, such as ulcerative colitis and Crohn's disease.

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Disclosures

The authors have no financial conflict of interest.

References

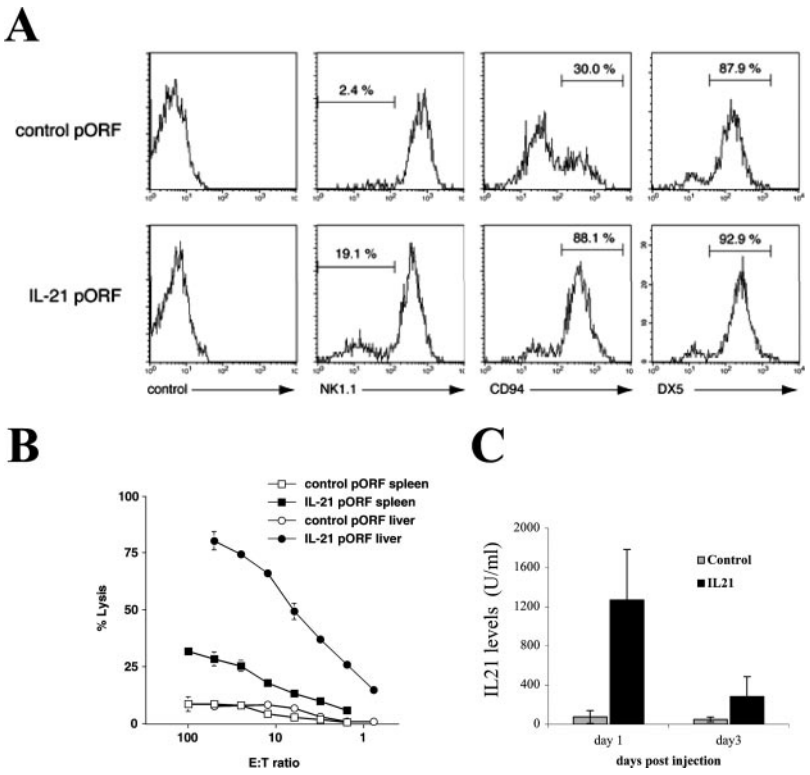
1. Isolauri, E., S. Salminen, and A. C. Ouwehand. 2004. Microbial-gut interactions in health and disease: probiotics. *Best Pract. Res. Clin. Gastroenterol.* 18: 299–313.
2. Sansonetti, P. J. 2004. War and peace at mucosal surfaces. *Nat. Rev. Immunol.* 4: 953–964.
3. Mahida, Y. R. 2004. Microbial-gut interactions in health and disease: epithelial cell responses. *Best Pract. Res. Clin. Gastroenterol.* 18: 241–253.
4. Rakoff-Nahoum, S., J. Paglino, F. Eslami-Varzaneh, S. Edberg, and R. Medzhitov. 2004. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell* 118: 229–241.
5. McCracken, V. J., and R. G. Lorenz. 2001. The gastrointestinal ecosystem: a precarious alliance among epithelium, immunity and microbiota. *Cell Microbiol.* 3: 1–11.
6. Gionchetti, P., F. Rizzello, U. Helwig, A. Venturi, K. M. Lammers, P. Brigidi, B. Vitali, G. Poggioli, M. Miglioli, and M. Campieri. 2003. Prophylaxis of pouchitis onset with probiotic therapy: a double-blind, placebo-controlled trial. *Gastroenterology* 124: 1202–1209.
7. Cui, H. H., C. L. Chen, J. D. Wang, Y. J. Yang, Y. Cun, J. B. Wu, Y. H. Liu, H. L. Dan, Y. T. Jian, and X. Q. Chen. 2004. Effect of probiotic on intestinal mucosa of patients with ulcerative colitis. *World J. Gastroenterol.* 10: 1521–1525.
8. Borruel, N., M. Carol, F. Casellas, M. Antolin, F. de Lara, E. Espin, J. Naval, F. Guarner, and J.R. Malagelada. 2002. Increased mucosal tumour necrosis factor α production in Crohn's disease can be downregulated ex vivo by probiotic bacteria. *Gut* 51: 659–664.
9. Borruel, N., F. Casellas, M. Antolin, M. Llopis, M. Carol, E. Espiin, J. Naval, F. Guarner, and J. R. Malagelada. 2003. Effects of nonpathogenic bacteria on cytokine secretion by human intestinal mucosa. *Am. J. Gastroenterol.* 98: 865–870.
10. Drakes, M., T. Blanchard, and S. Czinn. 2004. Bacterial probiotic modulation of dendritic cells. *Infect. Immun.* 72: 3299–3309.
11. Christensen, H. R., H. Frokiaer, and J. J. Pestka. 2002. Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells. *J. Immunol.* 168: 171–178.
12. Jijon, H., J. Backer, H. Diaz, H. Yeung, D. Thiel, C. McKaigney, C. De Simone, and K. Madsen. 2004. DNA from probiotic bacteria modulates murine and human epithelial and immune function. *Gastroenterology* 126: 1358–1373.
13. Neish, A. S., A. T. Gewirtz, H. Zeng, A. N. Young, M. E. Hobert, V. Karmali, A. S. Rao, and J. L. Madara. 2000. Prokaryotic regulation of epithelial responses by inhibition of IκB-α ubiquitination. *Science* 289: 1560–1563.
14. Collier-Hyams, L. S., H. Zeng, J. Sun, A. D. Tomlinson, Z. Q. Bao, H. Chen, J. L. Madara, K. Orth, and A. S. Neish. 2002. Salmonella AvrA effector inhibits the key proinflammatory, anti-apoptotic NF-κB pathway. *J. Immunol.* 169: 2 846–2850.
15. Hooper, L. V., M. H. Wong, A. Thelin, L. Hansson, P. G. Falk, and J. I. Gordon. 2001. Molecular analysis of commensal host-microbial relationships in the intestine. *Science* 291: 881–884.
16. Stappenbeck, T. S., L. V. Hooper, and J. I. Gordon. 2002. Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. *Proc. Natl. Acad. Sci. USA* 99: 15451–15455.
17. Sansonetti, P. J. 2001. Rupture, invasion and inflammatory destruction of the intestinal barrier by *Shigella*, making sense of prokaryote-eukaryote cross-talks. *FEMS Microbiol. Rev.* 25: 3–14.
18. Philpott, D. J., S. Yamaoka, A. Israel, and P. J. Sansonetti. 2000. Invasive *Shigella flexneri* activates NF-κB through a lipopolysaccharide-dependent innate intracellular response and leads to IL-8 expression in epithelial cells. *J. Immunol.* 165: 903–914.
19. Girardin, S. E., R. Tournebize, M. Mavris, A. L. Page, X. Li, G. R. Stark, J. Bertin, P. S. DiStefano, M. Yaniv, P. J. Sansonetti, and D. J. Philpott. 2001. CARD4/Nod1 mediates NF-kappaB and JNK activation by invasive *Shigella flexneri*. *EMBO Rep.* 2: 736–742.
20. Girardin, S. E., I. G. Boneca, L. A. Carneiro, A. Antignac, M. Jehanno, J. Viala, K. Tedin, M. K. Taha, A. Labigne, U. Zahringer, et al. 2003. Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. *Science* 300: 1584–1587.
21. Clerc, P., and P. J. Sansonetti. 1987. Entry of *Shigella flexneri* into HeLa cells: evidence for directed phagocytosis involving actin polymerization and myosin accumulation. *Infect. Immun.* 55: 2681–2688.
22. Petit-Bertron A.-F., T. Pedron, U. Gross, J.-Y. Coppee, P. J. Sansonetti, J.-M. Cavaillon, and M. Adib-Conquy. 2005. Adherence modifies the regulation of gene expression induced by interleukin-10. *Cytokine* 29: 1–12.

23. Li, C., and W. H. Wong. 2001. Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. *Proc. Natl. Acad. Sci. USA* 98: 31–36.
24. Pedron, T., C. Thibault, and P. J. Sansonetti. 2003. The invasive phenotype of *Shigella flexneri* directs a distinct gene expression pattern in the human intestinal epithelial cell line Caco-2. *J. Biol. Chem.* 278: 33878–33886.
25. Kufer, T. A., J. H. Fritz, and D. J. Philpott. 2005. NACHT-LRR proteins (NLRs) in bacterial Infect. *Immun. Trends Microbiol.* 13: 381–388.
26. Tschopp, J., F. Martinon, and K. Burns. 2003. NALPS: a novel protein family involved in inflammation. *Nat. Rev. Mol. Cell Biol.* 4: 95–104.
27. Goke, R., C. Gregel, A. Goke, R. Arnold, H. Schmidt, and B. Lankat-Buttgereit. 2004. Programmed cell death protein 4 (PDCD4) acts as a tumor suppressor in neuroendocrine tumor cells. *Ann. NY Acad. Sci.* 1014: 220–221.
28. Chapat, L., K. Chemin, B. Dubois, R. Bourdet-Sicard, and D. Kaiserlian. 2004. Lactobacillus casei reduces $CD8^+$ T cell-mediated skin inflammation. *Eur. J. Immunol.* 34: 2520–2528.
29. Kelly, D., J. I. Campbell, T. P. King, G. Grant, E. A. Jansson, A. G. Coutts, S. Pettersson, and S. Conway. 2004. Commensal anaerobic gut bacteria attenuate inflammation by regulating nuclear-cytoplasmic shuttling of PPAR-γ and RelA. *Nat. Immunol.* 5: 104–112.
30. Bambou, J. C., A. Giraud, S. Menard, B. Begue, S. Rakotobe, M. Heyman, F. Taddei, N. Cerf-Bensussan, and V. Gaboriau-Routhiau. 2004. In vitro and ex vivo activation of the TLR5 signaling pathway in intestinal epithelial cells by a commensal *Escherichia coli* strain. *J. Biol. Chem.* 279: 42984–42992.
31. Orlandi, R., M. Cattaneo, F. Troglio, P. Casalini, C. Ronchini, S. Menard, and I. Biunno. 2002. SEL1L expression decreases breast tumor cell aggressiveness in vivo and in vitro. *Cancer Res.* 62: 567–574.
32. Udono, H., D. L. Levey, and P. K. Srivastava. 1994. Cellular requirements for tumor-specific immunity elicited by heat shock proteins: tumor rejection antigen gp96 primes $CD8^+$ T cells in vivo. *Proc. Natl. Acad. Sci. USA* 91: 3077–3081.
33. Baker-LePain, J. C., M. Sarzotti, T. A. Fields, C. Y. Li, and C. V. Nicchitta. 2002. GRP94 (gp96) and GRP94 N-terminal geldanamycin binding domain elicit tissue nonrestricted tumor suppression. *J. Exp. Med.* 196: 1447–1459.
34. Bussolati, B., A. Ahmed, H. Pemberton, R. C. Landis, F. Di Carlo, D. O. Haskard, and J. C. Mason. 2004. Bifunctional role for VEGF-induced heme oxygenase-1 in vivo: induction of angiogenesis and inhibition of leukocytic infiltration. *Blood* 103: 761–766.
35. Skinner, H. D., J. Z. Zheng, J. Fang, F. Agani, and B. H. Jiang. 2004. Vascular endothelial growth factor transcriptional activation is mediated by hypoxia-inducible factor 1α, HDM2, and p70S6K1 in response to phosphatidylinositol 3-kinase/AKT signaling. *J. Biol. Chem.* 279: 45643–45651.
36. Ozawa, K., K. Kuwabara, M. Tamatani, K. Takatsuji, Y. Tsukamoto, S. Kaneda, H. Yanagi, D. M. Stern, Y. Eguchi, Y. Tsujimoto, et al. 1999. 150-kDa oxygen-regulated protein (ORP150) suppresses hypoxia-induced apoptotic cell death. *J. Biol. Chem.* 274: 6397–6404.
37. Shoshani, T., A. Faerman, I. Mett, E. Zelin, T. Tenne, S. Gorodin, Y. Moshel, S. Elbaz, A. Budanov, A. Chajut, et al. 2002. Identification of a novel hypoxia-inducible factor 1-responsive gene, RTP801, involved in apoptosis. *Mol. Cell. Biol.* 22: 2283–2293.
38. Ben-Neriah, Y. 2002. Regulatory functions of ubiquitination in the immune system. *Nat. Immunol.* 3: 20–26.
39. Tanaka, K., T. Kawakami, K. Tateishi, H. Yashiroda, and T. Chiba. 2001. Control of IkappaBalpha proteolysis by the ubiquitin-proteasome pathway. *Biochimie* 83: 351–356.
40. Krek, W. 2000. VHL takes HIF's breath away. *Nat. Cell. Biol.* 2: E121–E123.
41. Kamura T., D. M. Koepp, M. N. Conrad, D. Skowyra, R. J. Moreland, O. Iliopoulos, W. S. Lane, W. G. Kaelin, Jr., S. J. Elledge, R. C. Conaway, et al. 1999. Rbx1, a component of the VHL tumor suppressor complex and SCF ubiquitin ligase. *Science* 284: 657–661.

CORRECTIONS

Brady, J., Y. Hayakawa, M. J. Smyth, and S. L. Nutt. 2004. IL-21 induces the functional maturation of murine NK cells. *J. Immunol.* 172: 2048–2058.

Figure 8 is incorrect. The corrected figure is shown below.



Shurin, G. V., R. Ferris, I. L. Tourkova, L. Perez, A. Lokshin, L. Balkir, B. Collins, G. S. Chatta, and M. R. Shurin. 2005. Loss of new chemokine CXCL14 in tumor tissue is associated with low infiltration by dendritic cells (DC), while restoration of human CXCL14 expression in tumor cells causes attraction of DC both in vitro and in vivo. *J. Immunol.* 174: 5490–5498.

The second author's middle initial was omitted. The correct name is Robert L. Ferris.

Santiago, H. C., C. G. Feng, A. Bafica, E. Roffe, R. M. Arantes, A. Cheever, G. Taylor, L. Q. Vierira, J. Aliberti, R. T. Gazzinelli, and A. Sher. 2005. Mice deficient in LRG-47 display enhanced susceptibility to *Trypanosoma cruzi* infection associated with defective hemopoiesis and intracellular control of parasite growth. *J. Immunol.* 175: 8165–8172.

The eighth author's last name was misspelled. The correct name is Leda Q. Vieira.

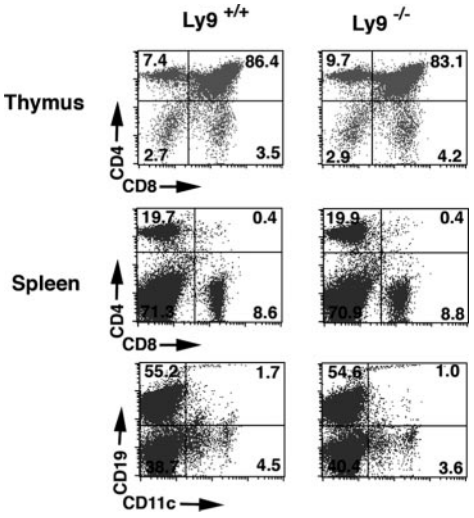
0022-1767/06/$02.00

Oki, T., J. Kitaura, K. Eto, Y. Lu, M. Maeda-Yamamoto, N. Inagaki, H. Nagai, Y. Yamanishi, H. Nakajina, H. Kumagai, and T. Kitamura. 2006. Integrin $\alpha_{IIb}\beta_3$ induces the adhesion and activation of mast cells through interaction with fibrinogen. *J. Immunol.* 176: 52–60.

The ninth author's last name was misspelled. The correct name is Hideaki Nakajima.

Graham, D. B., M. P. Bell, M. M. McCausland, C. J. Huntoon, J. van Deursen, W. A. Faubion, S. Crotty, and D. J. McKean. 2006. Ly9 (CD229)-deficient mice exhibit T cell defects yet do not share several phenotypic characteristics associated with SLAM- and SAP-deficient mice. *J. Immunol.* 176: 291–300.

In Figure 2A, the three left hand dot plot panels from Ly9+/+ cells were mistakenly duplicated in the three right hand dot plot panels of Ly9-/- cells. The numbers in each of the quadrants are correct and the error does not change any interpretation in the article. The corrected figure is shown below.



Tien, M.-T., S. E. Girardin, B. Regnault, L. Le Bourhis, M.-A. Dillies, J.-Y. Coppée, R. Bourdet-Sicard, P. J. Sansonetti, and T. Pédron. 2006. Anti-inflammatory effect of *Lactobacillus casei* on *Shigella*-infected human intestinal epithelial cells. *J. Immunol.* 176: 1228–1237.

One of the first author's affiliations was omitted. The corrected list of authors and affiliations is shown below.

Meng-Tsung Tien,[2]*†‡ Stephen E. Girardin,[2]* Béatrice Regnault,[†] Lionel Le Bourhis,[§] Marie-Agnès Dillies,[†] Jean-Yves Coppée,[†] Raphaëlle Bourdet-Sicard,[¶] Philippe J. Sansonetti,[3]* and Thierry Pédron*

*Pathogénie Microbienne Moléculaire Unit, Institut National de la Santé et de la Recherche Médicale U389, Paris France; [†]DNA Chip Platform, Genopole, Evry, France; [‡]Department of Biological Science and Technology, National Chiao Tung University, Hsinchu, Taiwan, China; [§]Imunité Innée et Signalisation, Pasteur Institute, Paris, France; and [¶]Danone Vitapole, Nutrivaleur, Palaiseau, France

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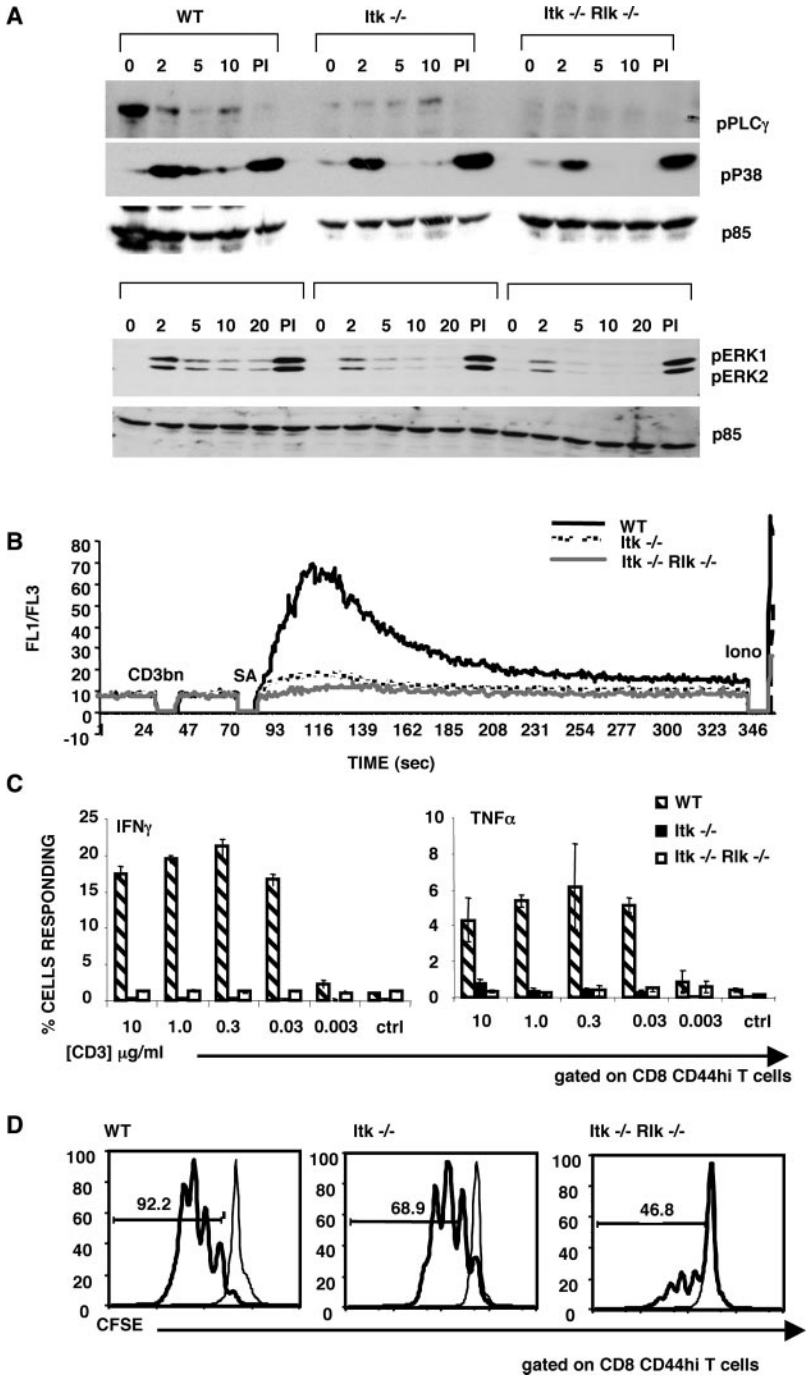
Atherly, L. O., M. A. Brehm, R. M. Welsh, and L. J. Berg. 2006. Tec kinases Itk and Rlk are required for $CD8^+$ T cell responses to virus infection independent of their role in $CD4^+$ T cell help. *J. Immunol.* 176: 1571–1581.

In Figure 1B, the WT Ca flux data line is missing from the Ca flux graph. The corrected figure is shown below.



Stone, J. D., and L. J. Stern. 2006. CD8 T cells, like CD4 T cells, are triggered by multivalent engagement of TCRs by MHC-peptide ligands but not by monovalent engagement. *J. Immunol.* 176: 1498–1505.

In **Discussion**, the last reference in the paper is incorrect. The corrected sentence and reference are shown below.

It is known that the cytoplasmic domains of several components of the TCR complex tend to homo-oligomerize at high concentrations (41); perhaps ligand-induced clustering of the TCR drives the cytoplasmic domains of proximal receptors to rearrange, exposing the Nck binding epitope and propelling other signaling cascade processes.

41. Sigalov A., D. Aivazian, and L. Stern. 2004. Homooligomerization of the cytoplasmic domain of the T cell receptor zeta chain and of other proteins containing the immunoreceptor tyrosine-based activation motif. *Biochemistry* 43: 2049–61.

Serhan, C. N., K. Gotlinger, S. Hong, Y. Lu, J. Siegelman, T. Baer, R. Yang, S. P. Colgan, and N. A. Petasis. 2006. Anti-inflammatory actions of neuroprotectin D1/protectin D1 and its natural stereoisomers: assignments of dihydroxy-containing docosatrienes. *J. Immunol.* 176: 1848–1859.

In **Discussion**, in the second sentence of paragraph six, 10*S*-HDNA should have been 10*S*-HDHA. The corrected sentence is shown below.

Recently, classic steric analysis of 10*S*-HDHA and the formation of 10,20-diHDHA and 17-H(p)DHA were reportedly optimized for the plant LOs (49).

Canté-Barrett, K., E. M. Gallo, M. M. Winslow, and G. R. Crabtree. 2006. Thymocyte negative selection is mediated by protein kinase C- and Ca^{2+}-dependent transcriptional induction of Bim of cell death. *J. Immunol.* 176: 2299–2306.

The title of the article is incorrect. The corrected title is shown below. The error has been corrected in the online version, which now differs from the print version as originally published.

Thymocyte Negative Selection Is Mediated by Protein Kinase C- and Ca^{2+}-Dependent Transcriptional Induction of Bim