Genetic and pharmacological targeting of TPL-2 kinase ameliorates experimental colitis: a potential target for the treatment of Crohn's disease?

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Inflammatory bowel disease is characterized by dysregulated immune responses against intestinal microflora leading to marked activation of nuclear factor-κB (NF-κB) with subsequent production of pro-inflammatory cytokines. Besides NF-κB, the tumor progression locus 2 (TPL-2)/extracellular signal-regulated kinase (ERK) pathway also regulates inflammatory cytokines such as interleukin-1β and tumor necrosis factor-α, but its role during intestinal inflammation is incompletely understood. We analyzed the impact of TPL-2 in the dextran sulfate sodium-induced experimental colitis model. Despite normal activation of NF-κB, animals lacking TPL-2 developed only mild colitis with reduced synthesis of inflammatory cytokines. Further, pharmacological inhibition of the TPL-2 kinase was similarly effective in ameliorating colitis as TPL-2 deficiency without obvious side effects. Because increased TPL-2/ERK activation was seen in patients with Crohn's disease (CD) but not ulcerative colitis, our findings encourage further investigation of TPL-2 kinase as potential target for the treatment of CD patients.

INTRODUCTION

Inflammatory bowel diseases (IBDs), comprising Crohn's disease (CD), and ulcerative colitis (UC) are chronic inflammatory diseases of the gastrointestinal tract with increasing prevalence in the western world. In addition to a genetic predisposition, aberrant activation of innate and adaptive immune mechanisms by the commensal flora has been claimed to initiate and perpetuate IBD.[1-3] Interactions between microbiota and host are mediated through pattern recognition receptors, e.g., Toll-like receptors (TLR) and nucleotide-binding oligomerization domain-like receptors (NLR). Nuclear factor-κB (NF-κB) links TLR activation with the production of pro-inflammatory cytokines, chemokines, and adhesion molecules, which control the recruitment of immune cells to sites of inflammation and infection.[4] Tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) in turn strongly activate NF-κB, which has a central role in amplifying and extending the duration of the innate immune response.[5] Dysregulation of NF-κB leads to constant overexpression of pro-inflammatory cytokines and is associated with a number of chronic inflammatory disorders such as rheumatoid arthritis and IBD.[6-9] In addition to NF-κB, induction of pro-inflammatory

cytokines also involves activation of mitogen-activated protein (MAP) kinases. Tumor progression locus 2 (TPL-2) was originally identified as a proto-oncogene and functions as a MAP 3-kinase that phosphorylates and activates the extracellular signal-regulated kinase (ERK).[10,11] In unstimulated macrophages, TPL-2 is associated with the inhibitor of NF-κB (IκB), p105, and activation of the TPL-2/ERK cascade in response to lipopolysaccharide (LPS) stimulation involves IκB kinase induced release from p105, demonstrating that the IκB kinase (IKK) complex regulates both, NF-κB and ERK activation.[5]

TPL-2 deficiency causes a massively reduced production of TNF-α in LPS-stimulated macrophages and TPL-2$^{-/-}$ mice are resistant to LPS/D-galactosamine-induced shock.[12] In addition to TNF-α, TPL-2 regulates IL-1β production in macrophages and dendritic cells (DCs) demonstrating that TPL-2 is involved in the control of innate immune responses.[13]

Increased activation of NF-κB, associated with enhanced IL-1β, TNF-α, and IL-6 expression has been reported to correlate with the degree of inflammation in IBD.[8,9] Because TPL-2 is linked to NF-κB signaling, we studied the impact of this MAP kinase pathway on inflammation in the dextran

sulfate sodium (DSS)-induced experimental colitis model. Lack of TPL-2 resulted in diminished production of pro-inflammatory cytokines and consequently milder inflammation independent of NF-κB activation. Further, treatment with the TPL-2 kinase inhibitor efficiently reduced intestinal inflammation. Our findings not only emphasize the critical role of TPL-2 in regulation but also suggest TPL-2 kinase as promising target for the treatment of IBD.

RESULTS
Absence of TPL-2 ameliorates DSS colitis
The role of TPL-2 was analyzed in the DSS model of experimental colitis. Mice given DSS in drinking water develop acute colitis with loss of body weight, diarrhea, and shortening of the colon.[14] In contrast to wild-type (WT) controls, which lost up to 20% of their initial body weight, TPL-2$^{-/-}$ mice developed only mild intestinal inflammation with a maximal body weight loss of ~5% (**Figure 1a**). In addition, reduction in colon length was far less pronounced in TPL-2$^{-/-}$ mice as compared with WT controls (**Figure 1b**).

Histopathological analysis of the colon from WT revealed severe ulcerations with submucosal infiltrations, whereas only minor lesions with reconstitution of epithelial cells were observed in TPL-2$^{-/-}$ mice (**Figure 1c**). Less prominent tissue damage in TPL-2$^{-/-}$ mice as compared with WT controls resulted in a lower colitis score for TPL-2$^{-/-}$ mice (**Figure 1d**). These data suggest that in addition to NF-κB, signaling via the MAP kinase pathway contributes to inflammation during DSS-induced colitis.

Absence of TPL-2 from cells of hematopoietic origin ameliorates colitis
Intestinal inflammation involves several cell types including immune cells of hematopoietic origin as well as cells of the stroma and the intestinal epithelium. To define the cell types involved in TPL-2-mediated inflammation, we generated bone marrow (BM) chimeras of WT and TPL-2$^{-/-}$ mice. WT mice reconstituted with TPL-2$^{-/-}$ BM cells developed milder inflammation during DSS treatment as compared with controls, i.e., WT mice reconstituted with WT BM (**Figure 2a**). Reciprocally, TPL-2$^{-/-}$ mice reconstituted with WT BM cells developed more pronounced inflammation than TPL-2$^{-/-}$ mice reconstituted with TPL-2$^{-/-}$ BM cells (**Figure 2b**). These results indicate that deficiency of TPL-2 in hematopoietic cells is sufficient to reduce intestinal inflammation, whereas lack of TPL-2 from non-hematopoietic cells has no apparent influence on development of colitis.

To determine whether TPL-2 has any impact on repair mechanisms by epithelial cells, we assessed proliferation of intestinal epithelial cells. Staining of Ki-67 in colon sections of WT and TPL-2$^{-/-}$ mice revealed similar proliferation during DSS treatment (**Figure 2c**), suggesting that epithelial repair and barrier function are not influenced by TPL-2 signaling.

Taken together, the lack of TPL-2 in mucosal cells of hematopoietic origin, rather than in intestinal epithelial cells,

was responsible for reduced inflammation during DSS colitis as seen in TPL-2$^{-/-}$ mice.

TPL-2 has no major impact on inflammatory cell infiltration during DSS colitis
Massive infiltration of neutrophils and monocytes in inflamed tissues is characteristic for IBD pathogenesis.[15] Thus, we dissected the influence of TPL-2 on the cellular composition of infiltrates during colitis and measured chemokines attracting DCs, neutrophils, and monocytes in supernatants of colon cultures. Apparent differences in the chemokine pattern between TPL-2$^{-/-}$ and WT animals during DSS colitis were not observed, but a trend toward lower production of macrophage inflammatory protein-1α (MIP-1α) in the absence of TPL-2 was seen. In addition, granulocyte colony-stimulating factor (G-CSF), a growth factor for neutrophils, which is also responsible for DC recruitment, was reduced in mutant mice (**Figure 3a**). With respect to frequencies and total numbers of infiltrating cells, only slight differences in DCs, neutrophils, and macrophages were observed in the lamina propria of DSS-treated TPL-2$^{-/-}$ animals as compared with controls, suggesting that TPL-2 has only a minor impact on regulating the influx of immune cells to the site of inflammation (**Figure 3c–e**).

TPL-2 regulates cytokine production during DSS colitis
Having shown that infiltration of immune cells is only slightly affected by the lack of TPL-2, we next determined additional mechanisms by which TPL-2 contributes to enhanced inflammation during DSS treatment. To this end, we analyzed the impact of TPL-2 signaling on cytokine production.

Eight days after DSS treatment, colons of mutant and control animals were removed and cultured for 24 h. Subsequently, cytokines were measured in supernatants using multiplex technology. The abundance of IL-1β, IL-6, and TNF-α was reduced in TPL-2$^{-/-}$ mice as compared with WT animals (**Figure 4a**). These cytokines have a central role during inflammation, as they induce the production of additional pro-inflammatory cytokines. RNA expression of these cytokines revealed lower mRNA concentrations of IL-1β and IL-6 in the colon of TPL-2$^{-/-}$ mice, indicating that TPL-2 is involved in transcriptional regulation of these cytokines (**Figure 4b**). In contrast, transcription of TNF-α was normal in the absence of TPL-2 despite reduced protein abundance. This is in line with previous reports, showing that TPL-2 regulates TNF-α at multiple levels, including post-transcriptional and post-translational levels.[12,16] With respect to TPL-2-mediated regulation of IL-1β, IL-6, and TNF-α, comparable results in LPS-stimulated bone marrow macrophages (BMMs) demonstrate direct transcriptional regulation of IL-1β and IL-6 (**Figure 5a**). In addition, concentrations of IL-1α, IL-10, and IL-17 were markedly reduced in colon cultures of TPL-2$^{-/-}$ mice as compared with controls (**Figure 4c**). Our data suggest that TPL-2 regulates numerous pro-inflammatory cytokines during intestinal inflammation.
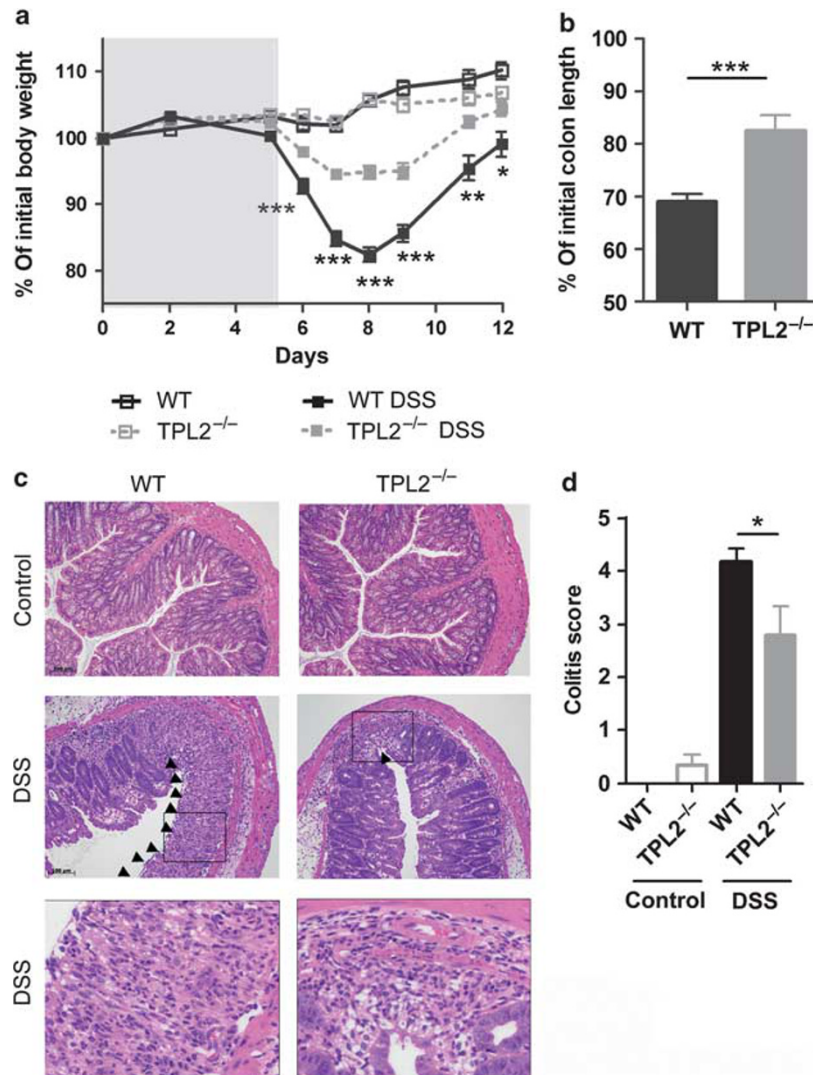
Figure 1 Absence of tumor progression locus 2 (TPL-2) ameliorates dextran sulfate sodium (DSS) colitis. Colitis was induced in wild-type (WT) and TPL-2$^{-/-}$ mice by adding 3% DSS to the drinking water for 5 days. (**a**) Body weight was monitored over 12 days. Graph shows percentage of body weight relative to initial body weight ($n=18$) from two independent experiments. Significant differences of DSS-treated WT vs. TPL-2$^{-/-}$ mice are indicated for every time point: ***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$ (Student's t-test). (**b**) At day 8, colons were removed and colon length was determined and expressed as percentage relative to controls ($n=18$) from two independent experiments; ***$P \leq 0.001$ (Student's t-test). (**c**) Row 1 and 2 show representative images of hematoxylin and eosin (H&E)-stained colon sections of DSS-treated and -untreated mice, which were used for scoring of inflammation. Ulcerations are indicated by arrowheads (×10). Row 3 shows tissues at higher magnification of above indicated sectors (×40). (**d**) Inflammation score according to histological analysis of H&E-stained paraffin-embedded colon sections was performed blinded in nine mice per group; *$P < 0.05$ (Student's t-test). All graphs show mean + s.e.m.

Normal NF-κB activity in TPL-2-deficient macrophages after LPS stimulation

NF-κB is the key transcription factor for IL-1β and IL-6 induction. Consequently, we determined NF-κB signaling in the absence of TPL-2. Therefore, we generated BMMs from TPL-2$^{-/-}$ and WT mice and stimulated cells with LPS for various time points. LPS- or TNF-α-induced activation of ERK was impaired in the absence of TPL-2, whereas phorbol 12-myristate-13-acetate (PMA)-induced phosphorylation of ERK was not affected (**Figure 5b**). Regarding activation of NF-κB, we found rapid degradation of IκB-α in WT and TPL-2$^{-/-}$ BMMs after LPS stimulation, whereas degradation of NF-κB1 p105 was

delayed in both mice as compared with IκB-α (data not shown). In addition, stimulation with LPS induced normal nuclear translocation of p50 and unaltered NF-κB-binding activity in TPL-2$^{-/-}$ BMMs (**Figure 5c**).

Amelioration of experimental colitis by a TPL-2 kinase inhibitor

TPL-2 is specifically activated after immune stimulation, raising the question whether this molecule represents a potential target for the treatment of colitis. Different protocols, all consisting of five treatments with TPL-2 kinase inhibitor were tested, including every day or every other day treatment, starting at
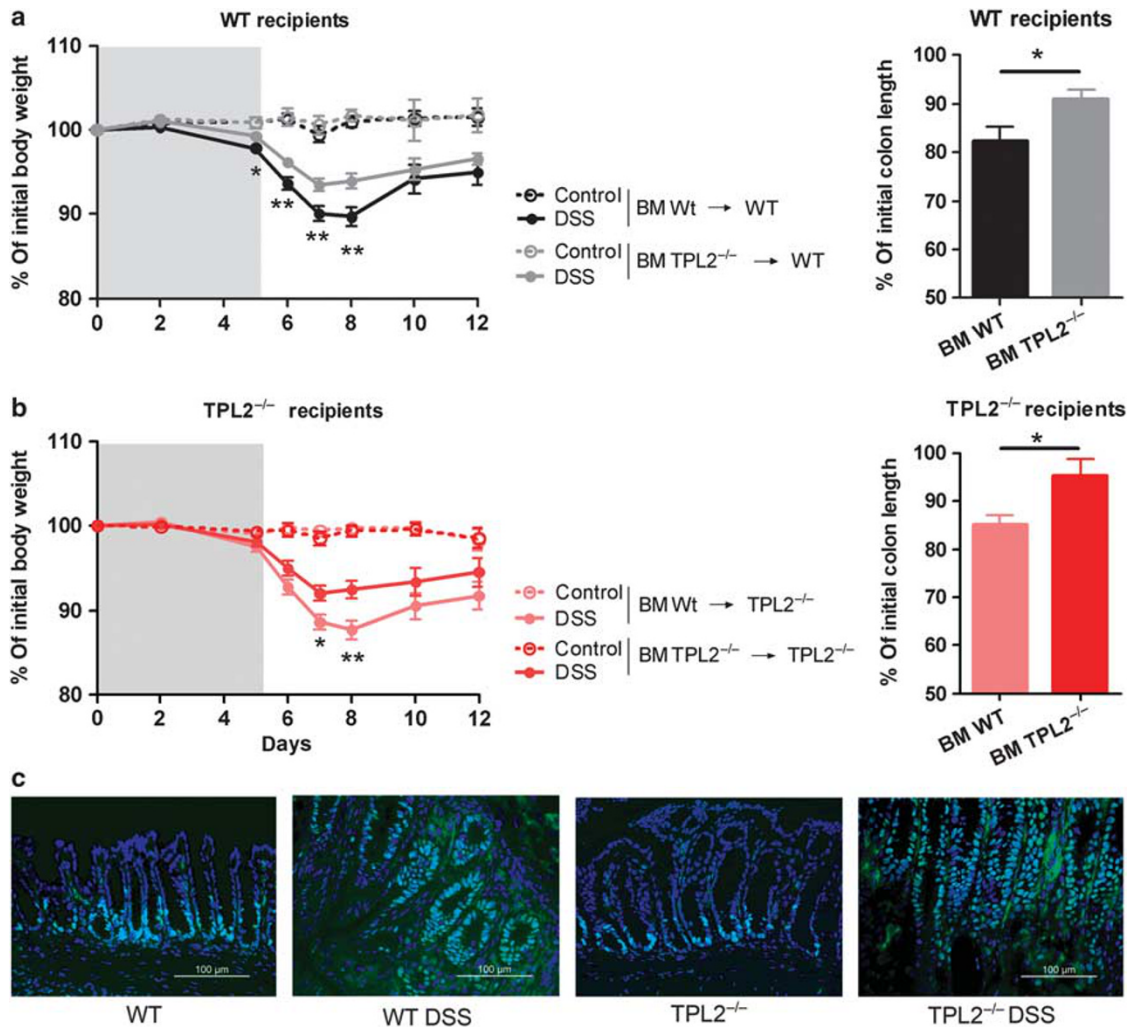
Figure 2 Lack of tumor progression locus 2 (TPL-2) in cells of hematopoietic origin ameliorates dextran sulfate sodium (DSS) colitis. Bone marrow (BM) chimeras of wild-type (WT) and TPL-2$^{-/-}$ mice were treated with 3% DSS in drinking water for 5 days. Body weight was monitored over 12 days during DSS colitis in controls ($n=12$) and DSS-treated mice ($n=18$). Pooled data from two independent experiments are shown. Significant differences in body weight change between the various DSS-treated BM chimeras are indicated: **$P \leq 0.01$; *$P \leq 0.05$ (Student's t-test). At day 8, colons were removed and colon length was determined. Graph shows percentage of colon length from DSS-treated ($n=11$) relative to untreated ($n=8$) controls; *$P \leq 0.05$ (Student's t-test). (**a**) Shown are WT recipients that received BM cells from TPL-2$^{-/-}$ or WT mice and (**b**) TPL-2$^{-/-}$ recipients that received BM cells from WT or TPL-2$^{-/-}$ mice. All graphs show mean + s.e.m. (**c**) Cryosections of the descending colon from WT and TPL-2$^{-/-}$ mice on days 0 and 8 of DSS colitis were stained for DNA (blue) and KI-67 (green) to monitor proliferating cells. Representative fluorescence images at ×40 magnification of five mice per group and time point are shown.

day 0 or day 2 of DSS colitis. Body weight was measured to monitor disease progression. Best results were obtained by daily inhibitor treatment of animals, starting at day 2 of DSS colitis. In addition, a control group only treated with TPL-2 kinase inhibitor revealed a similar kinetic of the body weight as untreated animals. Further, no macroscopic alterations of the gastrointestinal tract, the spleen, and the lymph nodes were detected, indicating that treatment with TPL-2 kinase inhibitor does not lead to obvious side effects (data not shown). As shown in **Figure 6b**, treatment with the TPL-2 kinase inhibitor efficiently reduced the loss of body weight and ameliorated colitis, as compared with vehicle-treated mice. Furthermore, analysis of ERK phosphorylation in BMMs revealed that inhibitor treatment reduced the amount of P-ERK after LPS stimulation (**Figure 6a**).

Finally, we wondered whether the TPL-2/ERK pathway is activated in IBD patients. Colonic samples of CD and UC patients were analyzed by western blot and compared with tumor patients who express constitutively activated ERK.[17] The data show that CD patients reveal enhanced activation of TPL-2/ERK as compared with UC patients (**Figure 7a,b**).

DISCUSSION

IBD is characterized by dysregulated immune responses to the intestinal microflora constantly monitored by TLRs present on epithelia and immune cells.[18] Stimulation of TLRs leads to NF-κB signaling, which causes production of pro-inflammatory cytokines. Prolonged and enhanced activation of NF-κB
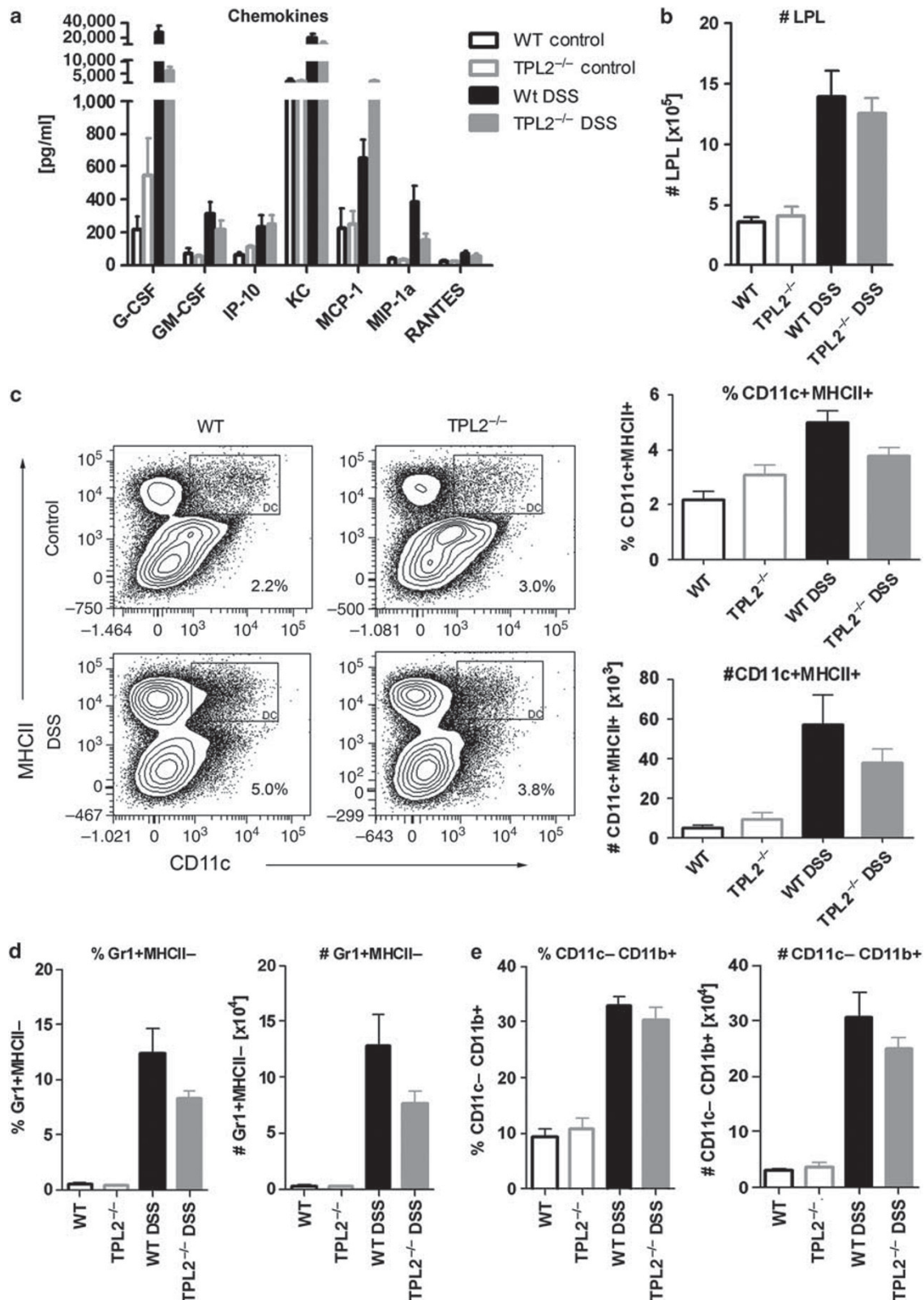
Figure 3 Tumor progression locus 2 (TPL-2) has a minor impact on inflammatory infiltrations during dextran sulfate sodium (DSS) colitis. Colons of DSS colitis were removed at day 8. (**a**) Sections of 1 cm from colons of DSS-treated (*n* = 7) and -untreated (*n* = 5) wild-type (WT) and TPL-2$^{-/-}$ mice were cultured *ex vivo* and chemokine secretion was measured in the supernatants using multiplex technology. (**b**) Alternatively, lamina propria leukocytes (LPLs) were isolated from naive and DSS-treated mice (*n* = 15). LPLs were stained with antibodies specific for Gr-1, MHC class II, CD11b, CD11c, and analyzed by flow cytometry. (**c**) Shown are representative frequencies and total numbers of dendritic cells, (**d**) neutrophils, (**e**) and macrophages from one out of three independent experiments. All graphs show mean + s.e.m. G-CSF, granulocyte colony-stimulating factor; MIP-1α, macrophage inflammatory protein 1α.
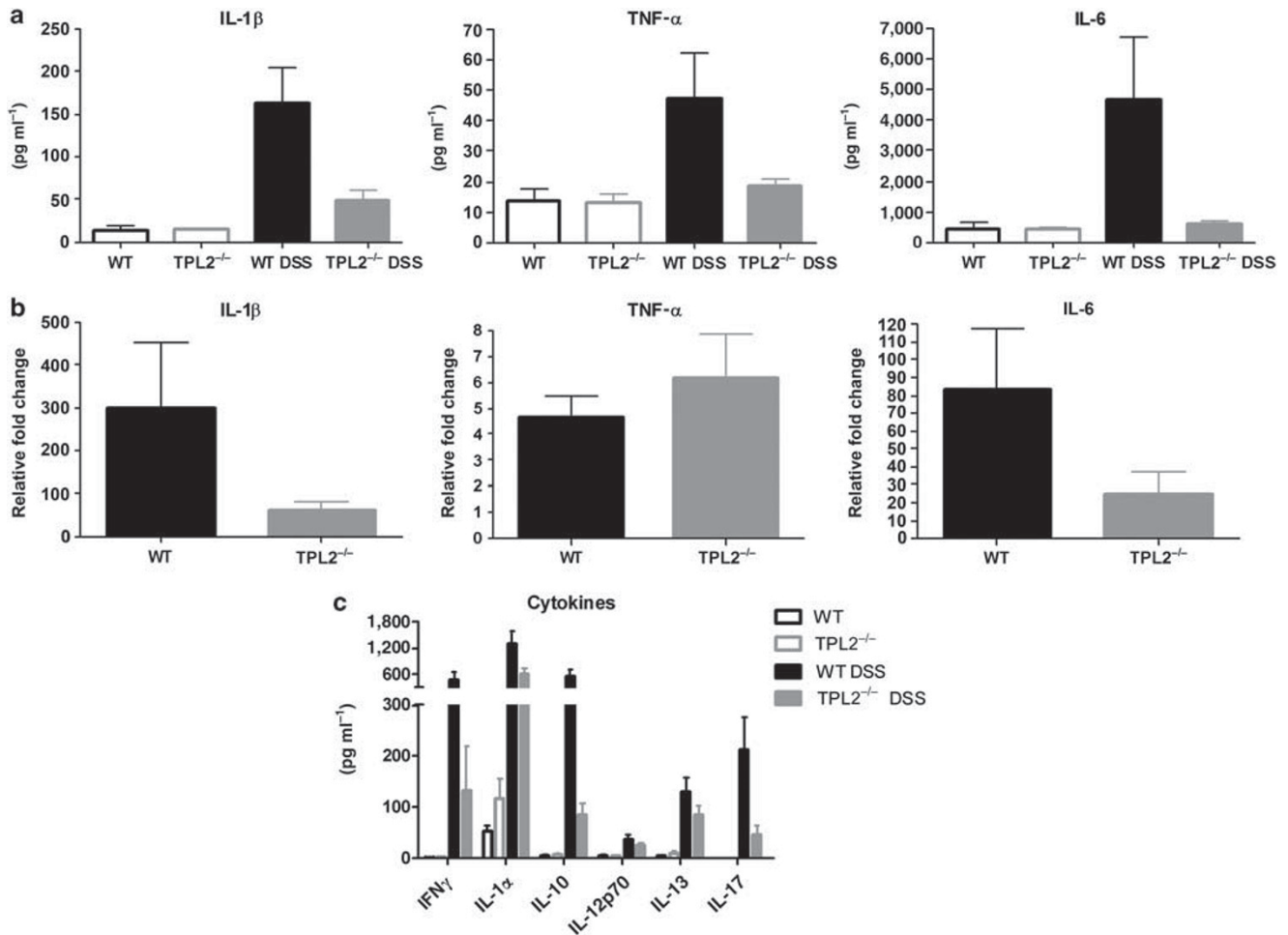
Figure 4 Absence of tumor progression locus 2 (TPL-2) inhibits cytokine production during dextran sulfate sodium (DSS) colitis. Colons were removed at day 8 of DSS colitis. (**a**, **c**) Cytokine secretion by colon explants from DSS-treated ($n=7$) and -untreated ($n=5$) wild-type (WT) and TPL-2$^{-/-}$ mice was measured in the supernatants using multiplex technology. (**b**) Alternatively, RNA was isolated from colons of DSS-treated and -untreated WT and TPL-2$^{-/-}$ mice ($n=5$) and cytokine expression was measured by real-time reverse transcriptase-PCR. All graphs show mean+s.e.m from one out of three independent experiments. IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

results in aberrant cytokine production and is associated with inflammatory disorders such as CD.[19,20]

In addition to NF-κB, the TPL-2/ERK MAP kinase pathway is involved in the synthesis of pro-inflammatory cytokines such as TNF-α and IL-1β.[12,13,16] However, in contrast to NF-κB, TPL-2 is exclusively activated by inflammatory stimuli rendering TPL-2 a promising target for anti-inflammatory therapy. Still, its role during intestinal inflammation is incompletely understood.

Our analysis of the role and biological function of TPL-2 in DSS-induced acute colitis revealed markedly reduced inflammation in absence of TPL-2.

It has been shown that $TNF^{\Delta ARE}$ mice develop T cell-dependent intestinal inflammation with sustained high levels of TNF-α. Lack of TPL-2 in these mice was shown to attenuate intestinal inflammation by affecting the pathogenic T-lymphocytic response.[21] In contrast, DSS colitis is T cell independent and mediated by macrophages.[22] Thus, both models demonstrate the crucial function of TPL-2

in regulating innate as well as adaptive immune responses during intestinal inflammation.

BM chimera experiments demonstrated the critical role in hematopoietic, but not stromal cells in TPL-2-mediated regulation of inflammatory responses during DSS colitis. Furthermore, we did not find an influence of TPL-2 on intestinal barrier and repair mechanisms.

IBD is characterized by massive infiltration of inflamed tissues by newly recruited blood neutrophils and monocytes, which produce pro-inflammatory cytokines.[15,23] Although DSS-treated TPL-2$^{-/-}$ mice revealed slightly reduced numbers of infiltrating DCs, macrophages, and neutrophils together with diminished amounts of G-CSF and MIP-1α, both responsible for activation and maturation of neutrophils, the biological significance of this observation remains currently unclear. As G-CSF has been shown to mobilize hematopoietic stem cells from the BM into the blood stream and to trigger DC recruitment in NOD mice,[24,25] we cannot exclude that low amounts of G-CSF is also responsible for reduced numbers of
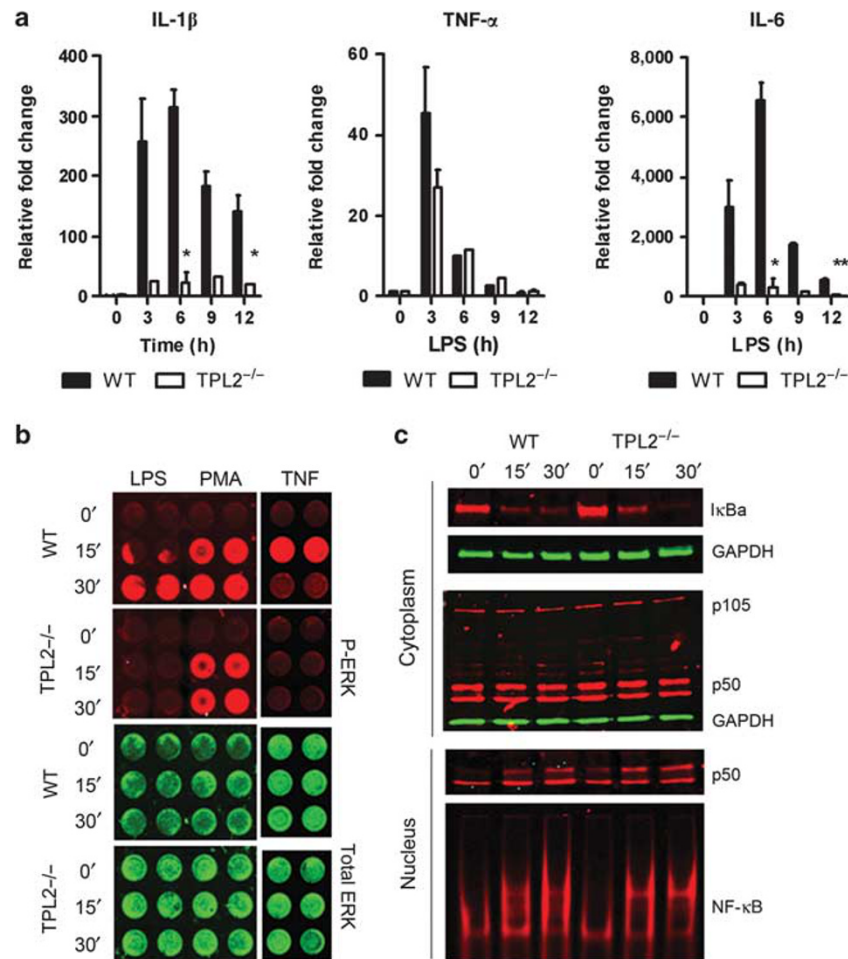
Figure 5 Normal nuclear factor-κB (NF-κB) activation in lipopolysaccharide (LPS)-stimulated tumor progression locus (TPL)-2$^{-/-}$ bone marrow macrophages (BMMs). BMMs were generated from wild-type (WT) and TPL-2$^{-/-}$ mice. Cells were stimulated with 100 ng ml^{-1} LPS at indicated time points. (**a**) RNA was isolated and cytokine expression of interleukin (IL)-1β, tumor necrosis factor-α (TNF-α), and IL-6 was measured by real-time reverse transcriptase-PCR ($n=2$). Graphs show mean + s.e.m, asterisks indicate significant differences of LPS-treated WT vs. TPL-2$^{-/-}$ cells; **$P<0.01$; *$P<0.05$ (Student's t-test). (**b**) For In-cell western blot assay, BMMs were stimulated in 96-well plates with LPS, PMA (100 nM), or TNF-α (100 ng ml^{-1}). Cells were fixed and incubated with antibodies against P-ERK (red) and total ERK (green). (**c**) Fluorescence western blot analysis was performed on cytosolic and nuclear extracts using antibodies against IκB-α, p105/p50 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). In addition, electrophoretic mobility shift assay was performed using a fluorescently labeled probe for NF-κB. One of three representative experiments is shown. ERK, extracellular single-regulated kinase; IKBα, inhibitor of NF-κB.

DCs, which then may affect T cell activation in the later phase of DSS colitis.

Importantly, the synthesis of the inflammatory cytokines IL-1β, TNF-α, and IL-6 was reduced in TPL-2$^{-/-}$ mice as compared with WT controls, suggesting that inflammatory cells remain anergic in TPL-2$^{-/-}$ mice. The observed variability in TNF-α abundance could reflect differences of cellular infiltrates in the inflamed tissues, as TPL-2-mediated induction of TNF-α has been shown to be cell and stimulus specific.[12,13,16]

The role of IL-1β in intestinal inflammation remains controversial. Increased amounts of IL-1β in the lamina propria of IBD patients suggest a contribution to intestinal inflammation.[26] To the contrary, NLR family, pyrin domain containing 3 (*Nlrp3*) inflammasome-induced production of IL-1β was shown to protect against colitis, and polymorphisms leading to

decreased *Nlrp3* expression have been associated with increased risk for CD.[27,28] Thus, IL-1β seems to have opposing effects, which might depend on its local concentration. Intriguingly, absence of TPL-2 did not completely abolish IL-1β expression but prevented excessive production of this cytokine. Therefore, the observed low abundance of IL-1β correlated with reduced inflammation in DSS colitis.

Overall, we speculate that TPL-2 mediates its inflammatory effects mainly via macrophages and DCs by promoting the production of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, during DSS colitis. Since DSS colitis can also be induced in the absence of B and T cells, it is very unlikely that inflammatory cytokines produced by T cells contribute to this form of colitis. NF-κB is central for the transcriptional control of inflammatory cytokines and the NF-κB inhibitory protein p105 stabilizes TPL-2.[5,29] Activation of TPL-2 signaling involves
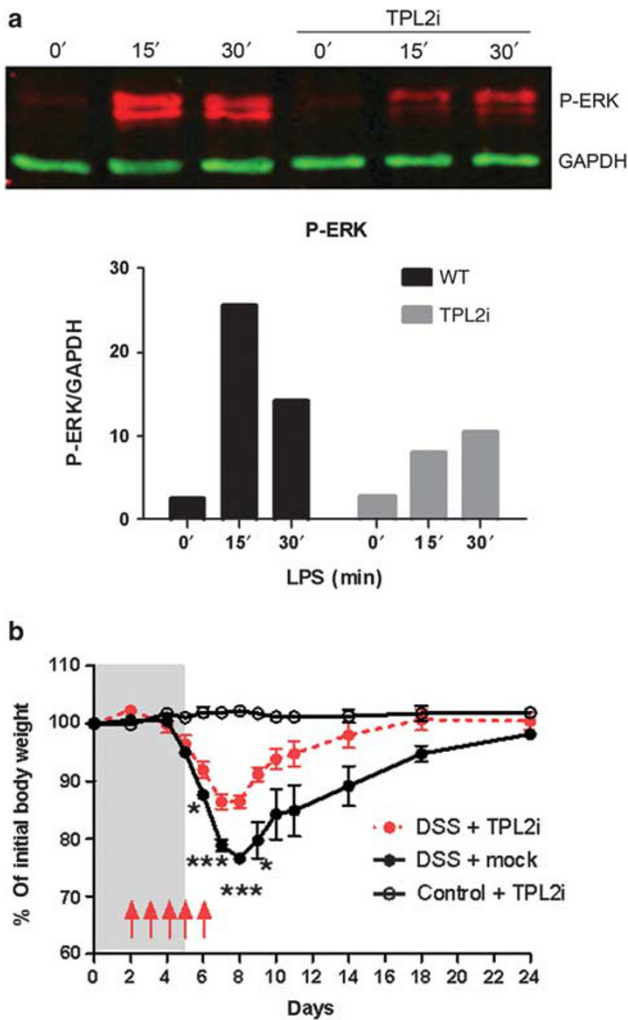
Figure 6 Treatment with tumor progression locus 2 (TPL-2) kinase inhibitor ameliorates dextran sulfate sodium (DSS) colitis. (**a**) Bone marrow macrophages were pre-treated with TPL-2 kinase inhibitor (TPL-2i, 20 μM) 1 h before lipopolysaccharide stimulation (100 ng ml⁻¹). Western blot analysis of total lysates using antibodies against P-extracellular signal-regulated kinase (P-ERK; red) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; green) and corresponding densitometry analysis of P-ERK relative to GAPDH. (**b**) Wild-type (WT) mice were treated with five doses of TPL-2 kinase inhibitor (50 μg per mouse) or vehicle (dimethyl sulfoxide) via the intraperitoneal route at indicated time points during DSS colitis. Body weight was monitored for 24 days ($n = 10$) in two independent experiments. All graphs show mean + s.e.m and data were analyzed by Student's t-test. Asterisks indicate significant differences of DSS-treated vehicle vs. TPL-2 kinase inhibitor-treated WT mice for every individual time point; ***$P < 0.001$; *$P < 0.05$. DSS, dextran sulfate sodium; LPS, lipopolysaccharide.

IKK-mediated release from p105 to activate its natural target the MAP kinase ERK.

Thus, both signaling pathways, NF-κB and TPL-2 are activated via the IKK kinase complex. However, although the transcription factor NF-κB regulates many cellular processes such as growth, survival, and immune responses, TPL-2 seems to be more specific and regulates only certain inflammatory cytokines during the immune response.

The example of TNF-α nicely demonstrates that both pathways act in concert to ensure the production of a functional protein: NF-κB initiates the transcription, whereas TPL-2 is needed for post-transcriptional and -translational modifications. This is in accordance with a recent study, which demonstrated that constitutive activation of NF-κB in the gut is not sufficient to induce destructive inflammation unless accompanied by activation of MAPK.[30]

In contrast, for IL-1β production, we still do not know the different roles of NF-κB and TPL-2. One possibility is that TPL-2 fine-tunes NF-κB at the level of p105. However, the impact of TPL-2 on p105 and NF-κB is currently incompletely understood because only a small portion of cellular p105 is associated with TPL-2.[31] Our experiments revealed comparable levels of p105 and p50 after LPS stimulation in TPL-2⁻/⁻ and WT BMMs, similar to TNF-α stimulation, which was previously published by Das et al.[32] Further, nuclear p50 and NF-κB-binding activity remained unaffected by the lack of TPL-2 in BMMs, suggesting that LPS triggered activation of NF-κB is normal in the absence of TPL-2. However, owing to the limited sensitivity of our analysis, we do not exclude that TPL-2 regulates the small pool of p105 to which it is physically attached.[31,32]

We were particularly interested in exploiting pharmacological inhibition of TPL-2 kinase for treatment of colitis,[31,33–36] notably as an alternative to treatment with anti-TNF antibodies, which is expensive and suitable for only a fraction of CD patients. The potential advantage of TPL-2 as therapeutic target is its selective activation by inflammatory stimuli with a consequent reduction in side effects. Thus, inhibition of TPL-2 will not affect activation of the MAP kinase ERK by other agonists, such as growth factors.

We demonstrate for the first time that treatment of mice with a TPL-2 kinase inhibitor ameliorated DSS colitis suggesting its potential for therapy of IBD patients. As a first step toward clinical application, we obtained evidence from a small cohort of IBD patients that the TPL-2/ERK pathway is active in CD but not UC patients. Increased P-ERK levels correlated with low TPL-2 protein levels, indicating that TPL-2 was activated and rapidly degraded afterward. Our data are consistent with a previous study showing elevated P-ERK levels in biopsies from CD as compared with UC patients.[37]

Selective activation of the TPL-2/ERK pathway in CD but not UC patients could be explained by differential proteasome composition in these two patient groups. As TPL-2 is linked to the inhibitory protein p105, its activation depends on proteasomal degradation of p105, which is accelerated in CD as compared with UC patients due to the presence of immunoproteasomes.[20] Thus, we assume that prolonged activation of NF-κB and TPL-2, as seen in CD patients, leads to a constant production of pro-inflammatory cytokines and consequently to chronic inflammation, while in UC patients, other pathways seem to be involved.

Amelioration of experimental colitis by blocking the TPL-2 kinase together with increased TPL-2/ERK signaling in CD patients point to this molecule as promising drug target for the treatment of CD.
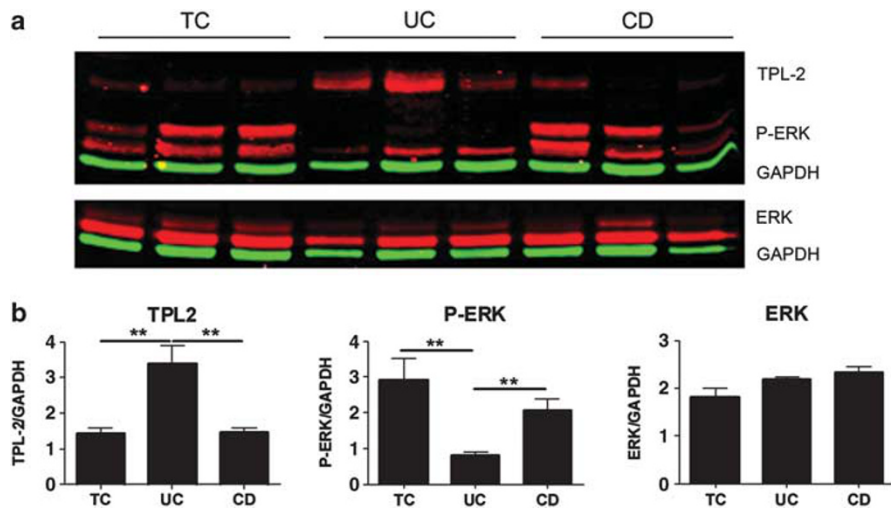
Figure 7 Tumor progression locus 2 (TPL-2)/extracellular signal-regulated kinase (ERK) is activated in patients with Crohn's disease. Human tissue samples from colon and small intestine from patients with CD, UC, or TC were immediately shock frozen after intestinal surgery. (**a**) A total of 20 µg of whole-cell lysates were analyzed by western blotting using antibodies against TPL-2, P-ERK, total ERK (red), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; green). (**b**) Graphs show densitometry analysis from western blots of TPL-2, P-ERK, and ERK relative to GAPDH for CD ($n=9$), UC ($n=8$), and TC ($n=8$) patients. Shown are mean+s.e.m. Asterisks indicate significant differences in TPL-2 and P-ERK levels between different patient groups; **$P<0.01$ (Student's t-test). CD, Crohn's disease; TC, tumor control; UC, ulcerative colitis.

METHODS

Patients. Human tissue samples were obtained from colonic specimens of patients undergoing surgery at the Charité University Hospital, Berlin. Tissues were immediately shock frozen and stored in liquid nitrogen. All patients provided informed consent and the study was approved by the local ethic committee of the Charité University Medical School, Berlin. A total of 25 patients with active CD (nine), UC (eight), and tumor patients as controls (eight) were investigated.

Mice. Mice were kept under special pathogen-free conditions at the animal facilities of the Max Planck Institute for Infection Biology. C57Bl/6 WT mice were obtained from Charles River Laboratories. TPL-2$^{-/-}$ mice were generated by P.N. Tsichlis and have been backcrossed nine times to C57Bl/6.[12] Animals were a generous gift of Anne O'Garra, National Institute for Medical Research, The Ridgeway, Mill Hill, London, England. For all studies, 10- to 12-week-old female WT and TPL-2$^{-/-}$ mice were used. Experiments were performed according to the German animal protection law.

DSS colitis. DSS colitis was assessed in WT and TPL-2$^{-/-}$ mice by administering 3% DSS salt reagent, MW 36,000–50,000 (MP Biomedical, Eschwege, Germany) for 5 days in drinking water. Body weight of individual mice was monitored and expressed as percentage relative to original body weight. At day 8, colons were removed and used for histopathology, colon culture, or isolation of lamina propria leukocytes. Histopathological analysis was performed on formalin-fixed colon sections, stained with hematoxylin and eosin. The DSS colitis score was assessed blinded by a pathologist and determined as a combination of inflammatory cell infiltration and tissue damage. Points for infiltration were given as follows: (0) no infiltration; (1) increased numbers of inflammatory cells in the lamina propia; (2) inflammatory cells extending into the submucosa; (3) transmural inflammatory infiltrates; and for tissue damage: (0) no mucosal damage; (1) discrete epithelial lesions; (2) erosions or focal ulcerations; (3) severe mucosal damage with extensive ulceration extending into the bowel wall.

Generation of BM chimera. Recipient mice were treated with ciprofloxacin hydrochloride (0.1 mg ml^{-1} in drinking water) before irradiation.

Mice were irradiated twice with 5 Gy with a 3-h time lag. One day later, 1×10^7 BM cells isolated from donor mice were injected intravenously. Mice were treated with ciprofloxacin hydrochloride (0.1 mg ml^{-1}) and neomycin (0.2 mg ml^{-1}) for 4 weeks. Colitis was induced 8–10 weeks after reconstitution.

Immunofluorescence staining. Immunofluorescence studies were performed using intestinal cryosections. Normal goat serum was applied to block nonspecific protein binding. Monoclonal rat anti-mouse Ki-67 antibody (Dako, Hamburg, Germany) was used, followed by Cy2 conjugated secondary antibody (Dianova, Hamburg, Germany). Images were acquired using a Leica DMRB microscope and ProgRes CapturePro 2.7 software (Jenoptik, Jena, Germany).

Treatment with TPL-2 kinase inhibitor. The TPL-2 kinase inhibitor is a cell-permeable naphthyridine compound that acts as a potent reversible and ATP-competitive inhibitor of TPL-2 kinase (Calbiochem, Darmstadt, Germany). Mice were treated with the TPL-2 kinase inhibitor (2.5 mg kg^{-1}, dissolved in dimethyl sulfoxide/phosphate-buffered saline) (Calbiochem) for a period of 5 days. TPL-2 kinase inhibitor was administered via the peritoneal route starting on day 2 of DSS colitis. Control mice were treated with dimethyl sulfoxide/phosphate-buffered saline (mock) at the same time points.

Cytokine analysis from *ex-vivo* colon culture. A section of 1 cm length of the descending colon was cultured in 1 ml RPMI (10% FCS, penicillin/streptomycin (P/S), 50 µg ml^{-1} gentamycin) for 24 h. Cytokines were measured in the supernatants using MILLIPLEX Cytokine/Chemokine kit according to manufacturer's instructions (Millipore, Billerica, MA).

Isolation of lamina propria leukocytes (LPL). Colons were removed, cut longitudinally, and washed in RPMI (10% FSC, 1 mM glutamin, P/S) for 30 min at 37 °C on a shaking platform. Intraepithelial mononuclear cells were removed by vigorous shaking in phosphate-buffered saline with 2% FCS for 30 s by hand. Colons were cut into small pieces and incubated in digestion medium with RPMI containing 0.4 mg ml^{-1} Collagenase VIII (125 collagenase digestive unit per mg; Sigma-Aldrich,

Munich, Germany) and 0.4 mg ml^{-1} Collagenase D (0.15 U mg^{-1}; Roche, Mannheim, Germany) for 1 h at 37 °C. Cell suspensions were filtered through a 130 μm iron mesh and centrifuged at 1,600 r.p.m. for 5 min. LPLs were recovered from the interphase of 40/70% Percoll gradient.

Flow cytometry analysis. For flow cytometry analysis staining, cells were blocked with rat serum and anti-Fc receptor. The following antibodies were used for staining: anti-MHCII-FITC (clone TIB120), anti-CD11c-Cy5 (clone N418), anti-Ly-6G-Pacific Blue (Gr-1) and anti-CD11b-PE-Cy7 (eBioscience, San Diego, CA). Cells were analyzed on the LSRII using FCS express 3 (*De Novo* Software, Los Angeles, CA). Briefly, lymphocytes were excluded in the forward scatter/side scatter plot. Neutrophils were defined as Gr1$^+$MHCII$^-$ cells and excluded from macrophage (CD11c$^-$CD11b$^+$) and DC (MHCII$^+$ CD11c$^+$) analysis.

Generation of BMMs. BM was obtained from 8- to 12-week-old female mice under sterile conditions. Cells were cultured in DMEM (10% FCS, 5% horse serum, 20% cell culture supernatant of macrophage colony-stimulating factor (CSF) produced by L929-CSF cells (MPIIB), 1 mM L-glutamine, 1 mM sodium-pyruvate, and 1× P/S) for 7 days.

RNA isolation and semi-quantitative real-time reverse transcriptase PCR. Colon sections were briefly incubated in 4 M guanidinium isothiocyanate and total RNA was extracted in TRIzol (Invitrogen, Darmstadt, Germany) using an Ultra Turrax. Pellets from BMMs were directly dissolved in TRIzol. RNA concentration and quality were determined by Agilent 2,100 Bioanalyzer (Agilent Technologies, Böttinger, Germany). Synthesis of complementary DNA was performed using the SuperScriptII Reverse Transcriptase (Invitrogen) according to manufacturer's instructions. Real-time reverse transcriptase PCR was performed using SYBR Green master mix (Applied Biosystems, Carlsbad, CA) with the ABI Prism 7000 sequence detection system using SDS2.2.2 Software (Applied Biosystems). Relative expressions were calculated with the ΔΔCt method using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. The following primers were used: GAPDH: 5′-CTCCACTCACGGCAAAT TCA-3′, 5′-GCCTCACCCCATTTGATGTT-3′; IL-1β: 5′-CAACC AACAAGTGATATTCTCCATG-3′, 5′-GATCCACACTCTCCA GCTGCA-3′; TNF-α: 5′-CATCCTCTCAAAATTCGAGTGAC AA-3′, 5′-TGGGAGTAGACAAGGTACAACCC-3′; IL-6: 5′-GAGG ATACCACTCCCAACAGACC-3′, 5′-AAGTGCATACTCGTTGTT CATACA-3′.

Preparation of total lysates from intestinal tissues. Frozen human colon samples were homogenized to powder with mortar and pestle and were dissolved in ice-cold lysis buffer containing 20 mM Tris-HCl pH 7.2, 50 mM NaCl, 0.1 % (v/v) NP-40, complete protease inhibitor cocktail (Roche), 1.5 mM phenylmethylsulfonylfluoride (PMSF), 0.2 mM Na$_3$VO$_4$, 50 mM NaF, and 1 mM dithiothreitol (DTT). Protein concentration was determined by protein-assay solution (Bio-Rad, Munich, Germany) against bovine serum albumin as standard.

Preparation of cytosolic and nuclear extracts from BMMs. Cell pellets were homogenized using a glass douncer in ice-cold lysis buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl$_2$, 1 mM DTT, 1.5 mM PMSF, 20 mM NaF, 200 μM Na$_3$VO$_4$, and complete protease inhibitor cocktail (Roche). After 15 min on ice, 0.25% NP-40 was added for 20 min and samples were centrifuged at 10,000 g for 2 min. Supernatants were used as cytosolic extracts. Pellets were washed and lysed in extraction buffer containing 20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM MgCl$_2$, 0.2 mM EDTA, 1 mM DTT, 1.5 mM PMSF, 20 mM NaF, 200 μM Na$_3$VO$_4$, and protease inhibitor cocktail for 30 min. Finally, suspensions were centrifuged at 13,000 r.p.m. for 15 min. Supernatants contained nuclear fractions. Protein concentration was determined by protein-assay solution (Bio-Rad) against bovine serum albumin

as standard. Purity of nuclear fractions was validated by western blot using anti-GAPDH antibody. All nuclear fractions revealed negative signals, while cytosolic fractions were positive for GAPDH.

Two-color fluorescent western blot analysis. Western blot analylsis was performed with 20–30 μg of total or cytosolic lysates, or 5 μg of nuclear lysate in 1× Laemmli buffer. Odyssey Blocking Reagent (Lincoln, NE) was used for blocking and antibody dilution. Membranes were incubated with rabbit antibodies against P-ERK and IκB-α (Cell Signaling, Frankfurt am Main, Germany) and ERK-2 and TPL-2 (Santa Cruz, Santa Cruz, CA), NF-κB p50/p105 (eBioscience) over night at 4 °C. Next day, membranes were incubated with anti-mouse GAPDH antibody (Calbiochem), anti-rabbit IgG Alexa Fluor 680 (red)- and anti-mouse IgG IrDye 800 (green)-labeled secondary antibodies (Rockland, Gilbertsville, PA) for 1 h. Membranes were scanned with the Odyssey Infrared Imaging System (Licor Bioscience) and densitometry analysis was performed with Odyssey Image Analyzer Software Version 1.2 (Licor Bioscience). Band intensity was normalized to GAPDH.

In-cell western blot assay. BMMs were stimulated in 96-well plates, fixed in 3.7% formaldehyde and permeabilized with 0.1% Triton-X solution. Cells were blocked with Odyssey Blocking Reagent (Licor Bioscience) and incubated with anti-rabbit P-ERK, anti-mouse total ERK (Cell Signaling), anti-rabbit IgG Alexa Fluor 680, and anti-mouse IgG IrDye 800 (Rockland) antibodies for 1 h. For detection, the 96-well plate was scanned with the Odyssey Infrared Imaging System (Licor Bioscience).

Infrared electrophoresis mobility shift assay. Infrared Dye (IRDye) 700-labeled oligonucleotides for NF-κB (5′-AGTTGAGG GGACTTTCCCAGGC-3′; 5′-GCCTGGGAAAGTCCCCTCAACT-3′ were synthesized from Thermo Electron GmbH (Karlsruhe, Germany). Annealing was performed for 2 min at 95 °C. Electrophoresis mobility shift assay-binding reaction was performed using 4 μg of nuclear extracts and the electrophoresis mobility shift assay buffer kit according to manufacturer's instructions (Licor Bioscience). Protein–DNA complexes were separated on 5% native polyacrylamide gel. For imaging, glass plates containing the gel were scanned with the Odyssey Infrared Imaging System (Licor Bioscience).

Statistical analysis. Statistical analysis was performed using a Student's *t*-test. For body weight curves, each time point was analyzed individually. Calculations were performed using GraphPad Prism 5.0. Differences of $P \leq 0.05$ were considered to be significant.

DISCLOSURE
The authors declare no conflict of interest.

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