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TNFα promotes mucosal wound repair through enhanced Platelet Activating Factor Receptor signaling in the epithelium

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Abstract

Pathobiology of several chronic inflammatory disorders including ulcerative colitis and Crohn's disease is related to intermittent, spontaneous injury/ulceration of mucosal surfaces. Disease morbidity has been associated with pathologic release of the pro-inflammatory cytokine Tumor Necrosis Factor alpha (TNFα). In this report, we show that TNFα promotes intestinal mucosal repair through upregulation of the GPCR Platelet Activating Factor Receptor (PAFR) in the intestinal epithelium. Platelet Activating Factor (PAF) was increased in healing mucosal wounds and its engagement with epithelial PAFR leads to activation of epidermal growth factor receptor, Src and Rac1 signaling to promote wound closure. Consistent with these findings, delayed colonic mucosal repair was observed after administration of a neutralizing TNFα antibody and in mice lacking PAFR. These findings suggest that in the injured mucosa, the pro-inflammatory milieu containing TNFα and PAF sets the stage for reparative events mediated by PAFR signaling.

Keywords

PAF; PAFR; TNFα; wound healing; epithelial barrier; intestine; inflammation; Inflammatory Bowel Disease

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Author Contributions

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Introduction

The gastrointestinal epithelium plays a pivotal role in separating luminal antigens and pathogens from underlying tissues. Mucosal inflammatory disorders such as inflammatory bowel disease, are associated with epithelial barrier compromise and mucosal wounds. Efficient epithelial repair is important in ensuring resolution of inflammation and restoration of mucosal homeostasis[1]. It is now appreciated that a compromised epithelial barrier results in spatiotemporal recruitment of immune cells that release mediators which interact with the epithelium to orchestrate mucosal repair[2].

Pro-inflammatory cytokines, such as TNFα, have been shown to initiate the synthesis of mediators that not only inhibit the inflammatory cascade but also serve to dismantle it, resulting in restoration of tissue homeostasis[3]. Several of these endogenous mediators include proteins/peptides and bioactive lipids which activate signaling cascades downstream of G-protein coupled receptors (GPCRs) in the epithelium and immune cell populations, stimulating proliferation and migration of cells to orchestrate mucosal repair[4].

Platelet Activating Factor Receptor (PAFR) is a GPCR expressed in intestinal epithelial cells (IECs) and immune cells such as neutrophils, monocytes and macrophages[5]. The PAFR ligand, Platelet Activating Factor (PAF) is a bioactive phospholipid that is released into the injured sites early in the inflammatory response[6]. Levels of PAF and PAFR are known to be elevated in the mucosa of individuals with IBD and in rodent models of colitis[7–9]. The active mucosal inflammatory response is associated with increased pro-inflammatory cytokine TNFα. Contribution of these inflammatory mediators to mucosal repair is however not well understood. A recent study revealed that TNFα promotes mucosal healing in colitis by influencing colonic epithelial progenitor cell population[10].

The role of PAFR signaling in influencing intestinal mucosal wound repair is uclear, and contradictory functional effects have been reported[11, 12]. Here we show that TNFα upregulates intestinal epithelial expression of PAFR during mucosal repair, leading to enhanced PAF signaling and intestinal epithelial wound repair. Furthermore, the pro-inflammatory cytokine IFNγ potentiates TNFα effects on PAFR signaling and mucosal repair.

Results

TNFα increases Platelet Activating Factor Receptor expression through NF-κB signaling

Molecules involved in initiation of the resolution phase of inflammation have been implicated in activating G protein coupled receptor (GPCRs) signaling to promote proliferation and migration of cells and wound repair. Since IFNγ and TNFα levels are elevated within actively inflamed intestinal mucosa and IFNγ has been shown to induce cell surface expression of TNFα receptors in epithelial cells[13], we examined the effects of both these cytokines on GPCR expression using PCR arrays and a model intestinal epithelial cell line (IEC) SKCO15.

As shown in figure 1A, the epithelial GPCR, Platelet Activating Factor receptor (Gene: *PTAFR* for human and *Ptafr* for mouse) was markedly upregulated in SKCO15 after TNFα and IFNγ incubation for 24 hrs (121 ± 32-fold, p<0.001). To confirm this result, two model intestinal epithelia cell lines, SKCO15 and T84 were treated with TNFα or/and IFNγ for 24 hrs and *PTAFR* mRNA expression was assessed by qPCR. In SKCO15 cells *PTAFR* was increased after incubation with TNFα (4.3 ± 0.25-fold) or IFNγ (2.77 ± 0.17-fold) compared to untreated controls. Furthermore, a marked increase in *PTAFR* expression was noted after simultaneous incubation with both TNFα and IFNγ (18.77 ± 2.96-fold; p<0.001; Figure 1B). Similar results were obtained using T84 IECs (Figure S1A). These results using model IECs were also confirmed in cultures of human colon epithelial cells referred to as colonoids. Consistent with the above results, *PTAFR* mRNA was increased after incubation of human colonoids with TNFα (3.79 ± 1.12-fold) and combined treatment with IFNγ and TNFα (7.21 ± 1.39-fold; p<0.05; Figure 1C) compared to untreated controls. *PTAFR* is expressed in a number of cells other than IECs. To determine if treatment with TNFα and/or IFNγ increased *PTAFR* expression in a different cell type, we isolated peripheral human blood monocytes from healthy donors and cultured them in presence or absence of the aforementioned cytokines. None of the treatment conditions lead to a significant increase in *PTAFR* mRNA levels (Figure S1B). To further dissect the specific roles of TNFα and IFNγ in IEC *PTAFR* mRNA upregulation, a functional experiment using neutralizing antibodies against TNFα and IFNγ was performed. SKCO-15 cells were treated with TNFα and IFNγ and control IgG, TNFα and/or IFNγ neutralizing antibodies were added to the media. Interestingly, the TNFα antibody alone (0.97 ± 0.02-fold; p<0.001) or in combination with the IFNγ antibody (1.13 ± 0.08-fold; p<0.001) inhibited the IEC *PTAFR* mRNA upregulation triggered by these cytokines, while the IFNγ antibody had no effect (34.00 ± 2.94-fold) (Figure 1D). Since TNFα activates NF-κB signaling, we determined if this signaling cascade contributes to the increase in *PTAFR* mRNA after TNFα/IFNγ treatment of IECs. SKCO15 cells were incubated with TNFα and IFNγ for 24 hours in presence or absence of Flavopiridol (1 μM) or Bay 11–7082 (10 μM), two inhibitors that block TNFα induced NF-κB activation[14, 15]. As shown in figure 1E (left panel), both inhibitors blocked TNFα triggered NF-κB signaling as determined by a NF-κB luciferase promoter assay. Furthermore, the increase in *PTAFR* mRNA in response to TNFα and IFNγ incubation was abolished when cells were co-incubated with Flavopiridol (16.55 ± 2.06 - fold; p<0.001) or Bay 11–0782 (2.87 ± 0.34-fold; p<0.001). Apoptosis of cells incubated with these inhibitors was analyzed by the TUNEL assay, showing no significant increase in death with either (Figure S1C). To further demonstrate the role of NF-κB signaling in upregulating intestinal epithelial *PTAFR* mRNA expression downstream of TNFα treatment, p65 siRNA was used to downregulate this subunit in SKCO15 cells. As shown in figure 1F, p65 downregulation decreased TNFα and IFNγ induced upregulation of *PTAFR* mRNA. Finally, to clarify if other pro-inflammatory cytokines directly result in *PTAFR* mRNA upregulation we treated SKCO15 cells with IL-1b and IL6. As shown in Fig S1D, neither of these cytokines caused an increase in intestinal epithelial levels of *PTARF*.

These findings indicate that TNFα signaling upregulates *PTAFR* expression which is potentiated by IFNγ, the latter of which has been shown to upregulate TNFα receptor expression in epithelial cells.

PAFR its important for in-vivo intestinal mucosal wound healing

To corroborate the *in vitro* findings above and elucidate the relevance of PAFR in intestinal mucosal wound repair, mRNA from healing biopsy induced murine colonic mucosal wounds was isolated, and quantification of *Ptafr* mRNA was determined using qPCR. *Ptafr* was increased within 24 hours (Day 1) following injury (2.83 ± 0.26-fold; $p<0.001$; Figure 2A) relative to intact intestinal mucosa. The increase in *Ptafr* mRNA went back to levels similar to an unwounded state on Day 2 and 3 following injury. To further characterize *Ptafr* expression *in vivo*, intestinal mucosal wounds were isolated from C57BL/6J mice and *Ptafr* mRNA levels were evaluated by *in situ* hybridization (ISH). Increased *Ptafr* expression was detected on Day 1 in epithelial cells adjacent to the healing intestinal mucosal wounds (Fig 2B). To verify the contribution of *Ptafr* in promoting colonic mucosal wound repair, we analyzed mucosal wound healing in PAFR-deficient ($Ptafr^{-/-}$) mice compared to wild-type (C57BL/6J) mice using a colonic mucosal biopsy induced injury model. While $Ptafr^{-/-}$ mice showed no difference in body weight, colon length or crypt length compared to C57BL/6J mice (Figure S2), delayed mucosal wound healing was observed in $Ptafr^{-/-}$ mice compared to C57BL/6J mice 3 days post injury (34.74 ± 1.1, $Ptafr^{-/-}$; 47.12 ± 0.58, C57BL/6J; $p<0.001$; Figure 2C). Taken together, these results support an important role of PAFR in promoting colonic mucosal wound repair.

PAF promotes in-vitro intestinal epithelial wound repair

Intestinal mucosal wound repair is a highly regulated process orchestrated by a spatiotemporal release of pro-inflammatory and pro-repair mediators. Since the ligand for PAFR is an inflammatory lipid mediator PAF, we next analyzed PAF levels in healing colonic biopsy induced wounds by Mass Spectrometry. Increased PAF was detected on Day 2 after wounding (Figure 3A, 1.58 ± 0.13-fold; $p<0.05$). Functional effects of PAF on epithelial wound repair were examined in an *in vitro* scratch wound healing assay using SKCO15 IECs. A dose response study revealed increasing effects of PAF in promoting wound repair at concentrations of 100 nM, 1 μM and 10 μM (Figure S3A). Cells were then treated with 10 μM PAF for 24 hours and wound repair was monitored by live time lapse imaging. Administration of PAF significantly increased wound closure within 24 hours (Figure 3B, $p<0.001$). This effect was confirmed in another model IEC line, T84 (Figure S3B). To further implicate contribution of PAF/PAFR signaling in promoting wound repair, SKCO15 cells were treated with the selective PAFR inhibitor PCA 4248 and PAF. PCA 4248 dose response studies were performed to determine its effect on epithelial wound closure. Furthermore, apoptosis was analyzed by TUNEL staining (Figure S3C, D). Treatment with 10 μM PCA 4248 and 10 μM PAF reduced wound closure compared to treatment with PAF alone (Figure 3B). These findings suggest that the pro-repair effect of PAF was abrogated by selectively inhibiting PAFR signaling. We next evaluated the combined influence of TNFα and/or IFNγ and PAF on epithelial wound closure *in vitro*. Treatment of SKCO15 cells with TNFα (100 ng/ml) or IFNγ (100 ng/ml) promoted wound closure within 24 hours (Figure 3C; TNFα $p<0.001$, IFNγ $p<0.05$). Use of both cytokines further enhanced wound closure within 24 hours ($p<0.001$). Additionally, we demonstrate that PAF treatment further enhanced TNFα and IFNγ mediated epithelial wound closure (Figure 3C). To determine whether the pro-repair effect of TNFα and IFNγ is, in part, mediated by signaling downstream of the PAFR, healing wounds of SKCO15 (Figure 3D)

and primary cultured intestinal monolayers (Figure 3E) were pre-incubated with PCA 4248 and subsequently incubated with TNFα and IFNγ alone or with PAF. PCA 4248 treatment reduced the pro-repair effects of TNFα, IFNγ and PAF at 18 hours post injury ($p<0.001$) (Figure 3D and 3E). These findings suggest that the pro-repair properties of TNFα and IFNγ are, in part, mediated by PAFR signaling. Taken together, these data support an important role for PAF in promoting intestinal mucosal wound repair.

PAF promotes cell matrix adhesion turnover by EGFR signaling via ADAM10 activation.

Wound closure is mediated by cell proliferation and migration. We next analyzed the mechanisms by which PAF promotes epithelial signaling to influence cell migration. Given a report of crosstalk between PAFR and epidermal growth factor receptor (EGFR)[16], we examined whether PAF influences EGFR activation leading to downstream signaling and enhanced cell motility. EGFR transactivation by GPCRs requires metalloproteases that cleave EGFR ligands. In the context of PAFR, a Disintegrin and Metalloproteinase domain-containing protein 10 (ADAM10) has been shown to cleave transmembrane heparin-binding EGF (HBEGF) that binds to EGFR[17]. To assess if ADAM10 activity increases after PAF treatment, a fluorimetric activity assay was performed using migrating SKCO15 IECs. PAF treatment increased ADAM10 activity within 30 minutes compared to treatment with control media ($p<0.05$; Figure 4A). We next evaluated EGFR activation by analyzing EGFR (Y845) phosphorylation in healing epithelial scratched monolayers (SKCO15, Figure 4B) and primary cultured intestinal sparse monolayers (Figure 4C) treated with PAF. Immunoblotting revealed increased EGFR phosphorylation at 30 minutes after PAF treatment (Figure 4B and 4C, S4A and S4B). EGFR activation has been reported to initiate signaling pathways involved in regulation of cell migration. These signaling mediators include tyrosine kinase Src (Y416 phosphorylation) downstream of EGFR signaling leading to activation of cell matrix adhesion proteins including focal adhesion kinase (FAK), which regulates turnover of cell matrix adhesions and forward cell movement. In addition, Src activated by G-proteins also contributes to EGFR activation. Thus, to explore PAF induced Src activation, SKCO15 healing wounds were treated with PAF and analyzed by immunoblotting. Increased pSrc-Y416 was observed 30 minutes after PAF treatment compared to unstimulated cells (Figure 4B and 4C, S4A and S4B). Increased FAK phosphorylation at the major autophosphorylation site Y397 as well as the Src-dependent pFAK-Y861 phosphorylation was also detected (Figure 4B and 4C, S4A and S4B). Additionally, increased pFAK-Y861 was identified in focal contacts of the migrating epithelial cells at the leading edge of wounds after PAF exposure for 2 hours by immunofluorescence labeling (Figure 4D). Taken together, these results support a role of EGFR activation, Src and FAK phosphorylation in response to PAF.

PAF enhances Rac1 activation, and Reactive Oxygen Species generation, influencing cell matrix adhesion

Rac1 is a small GTPase implicated in actin cytoskeletal restructuring during epithelial cell migration and wound repair[18, 19]. Additionally, EGFR activation enhances Rac1 activation. Thus, to further identify the mechanism by which PAF promotes epithelial cell migration and wound repair, Rac1 activation in response to PAF incubation was evaluated. Increased

Rac1-GTP was observed in epithelial cells migrating to heal wounds when incubated with PAF (Figure 5A).

Activation of Rac1 has been shown to promote reactive oxygen species (ROS) generation by associating with the intestinal epithelial oxidase NOX1 which leads to oxidative modification of phosphatases that regulate cell matrix adhesion regulatory proteins[20, 21]. Src has been reported to increase NOX-1 dependent ROS generation[22]. ROS can enhance EGFR signaling, Src phosphorylation and ADAM10 activation[23, 24]. Thus, we next analyzed the effects of PAF on ROS generation in IECs migrating to heal wounds, using an intracellular redox-sensitive dye, Hyro-Cy3. PAF exposure increased ROS generation within 15 mins of treatment (2.9 ± 0.2 fold; $p<0.001$; Figure 5B, S5A). The formyl peptide receptor 1 (FPR1) agonist, fMLf was used as a positive control for ROS generation. As shown in figure 5B, ROS generation was most prominent in migrating IECs at the leading edge of the wound. Inhibition of PAFR signaling by incubation with PCA 4248 inhibited the increase in ROS generation. Additionally, pretreatment with the ROS scavenger N-acetyl-cysteine (NAC) abrogated PAF-induced ROS generation (Figure 5B). Since intracellular ROS signaling induces oxidative modification and inhibition of phosphatases that regulate FAK signaling in cell-matrix adhesions and forward cell movement, we next analyzed the influence of PAF incubation on the adhesion properties of epithelial cells. To assess the effects of PAF in the regulation of adhesive forces, we measured cell adhesion strength by analyzing the force required to detach the cells from the extracellular matrix. Epithelial cells were seeded on fibronectin-coated glass coverslips, allowed to adhere for 6 hours after which they were exposed to hydrodynamic shear forces using a spinning disk device. The number of adherent cells were counted at differing radial positions, corresponding to known shear stress values. The fraction of adherent cells decreases nonlinearly with respect to fluid shear stress. The cell adhesion strength was defined as the shear stress that produces 50% detachment of cells. PAF treatment of SKCO15 IECs significantly increased cell adhesion strength (Figure 5C, D; 228 dyn/cm^2 vs. 271 dyn/cm^2, $p<0.01$), suggesting that PAF regulates cell-matrix mechanical interactions. Additionally, cell spreading area was determined and focal adhesion (FA) size was analyzed by staining for vinculin, a FA protein important for FA assembly and force transmission. We did not observe a difference in cell spreading area (Figure S5B). In contrast, PAF treated SKCO15 IECs exhibited higher area of vinculin-containing FAs compared to control cells (4.3 ± 0.2 μm^2 vs 2.1 ± 0.1 μm^2; $p<0.001$; Figure 5E, F). We attributed the increased adhesion strength of PAF treated cells to increased FA area, as we have previously shown cell adhesion strength increases with FA area[25]. Taken together, these data indicate PAF regulates adhesion of epithelial cells to the matrix.

TNFα neutralization impairs intestinal wound repair

Epithelial cell damage and mucosal wounds are frequently seen in the mucosal lesions of individuals with IBD who have active disease. Although murine experimental colitis models consistently produce severe mucosal injury, understanding the mechanisms that control wound healing in these systems has been challenging because of the variable timing and location of wounds. We utilized a simple murine colonic mucosal biopsy induced wound healing model to investigate the kinetics of inflammatory response and mucosal repair. Analysis of healing biopsy-induced wounds revealed increased *Tnf* mRNA expression with

peak levels observed on day 1 after injury (4.26 ± 0.58-fold increase, p<0.001; Figure 6A). In contrast, significant changes in *Ifng* mRNA were not observed in healing wounds (Figure 6B). Our findings suggest that the pro-inflammatory cytokines IFNγ and TNFα contribute to epithelial wound repair, in part due to PAFR upregulation. Given that *Tnf* mRNA expression was increased *in vivo* after wounding, we investigated the influence of TNFα on wound repair by intraperitoneal (ip) administration of a neutralizing TNFα antibody (MP6-XT3), which has been shown to significantly diminish TNFα levels after a single ip injection[26]. Importantly, in contrast to IgG control antibody, *Ptafr* mRNA up-regulation was not observed in biopsy induced wounds from mice administered TNFα neutralizing antibody (Figure 6C).

There was a significant reduction in colonic mucosal wound closure in mice after anti-TNFα antibody treatment (23.56 ± 0.95%, antibody; 47.22 ± 1.34%, control; p<0.001; Figure 5D). Antibody neutralization efficiency was confirmed by detection of reduced mRNA levels of the TNFα target proteins *Il6* and *Cxcl-10* (Figure S6). Taken together these findings support an important role of TNFα in mucosal repair.

Discussion

Pro-inflammatory lipids and eicosanoids, leukotrienes, free radicals, and cytokines, including TNFα are released into injured sites during an inflammatory response. It is generally appreciated that TNFα plays an integral role in the pathogenesis of chronic inflammatory states such as inflammatory bowel disease; nevertheless evidence that this cytokine is important for the establishment of a repair phase has started to emerge.

In this work we observed that TNFα, in combination with IFNγ, has synergistic effects in upregulating *PTAFR* mRNA in IECs. This response seems to be specific as other pro-inflammatory cytokines as IL-1β and IL-6 do not replicate this phenomenon. Previous studies have shown that IFNγ activates STAT-1a and inhibits recruitment of this protein to the TNFα receptor 1, directing TNFα signaling towards NF-κB. Additionally, IFNγ treatment increases cell surface expression of TNFα receptor in epithelial cells[13]. A recent report suggested that TNFα promotes mucosal healing in colitis by activating NF-κB and Wnt/β-catenin signaling in colonic epithelial stem-progenitor cell population[10]. In our study, TNFα upregulates *PTAFR* mRNA downstream of NF-κB signaling. In contrast to its pro-inflammatory role in myeloid cells, NF-κB has a protective role in IEC by influencing intestinal epithelial homeostasis. Mice with conditional deletion of proteins of the NF-κB in IEC display chronic colitis[27, 28]. These studies suggest that balanced NF-κB signaling has beneficial effects and help to restore mucosal homeostasis.

We provide evidence for cross-talk between the pro-inflammatory cytokine TNFα and PAFR in the early phase of intestinal inflammation following epithelial injury that has beneficial effects on wound repair. In these studies, we observed *Ptafr* upregulation during the early inflammatory phase after injury and decreased wound healing in *Ptafr*$^{-/-}$ mice. These observations are supported by previous studies in which *Ptafr* deletion augmented skin inflammation in a chemically induced skin injury model[29]. Other studies showed a beneficial role of PAFR during pulmonary infections with *Klebsiella pneumoniae*[30]. *Ptafr*$^{-/-}$

mice exhibited decreased anaphylactic reactions, better outcome in graft-versus-host disease and delayed lethality following intestinal ischemia and reperfusion injury through reduced inflammatory responses[31–33]. Increased expression of PAFR and PAF has been reported in the mucosa of individuals with IBD and in rodent models of colitis. In these conditions, increased PAFR expression are likely the result of ongoing inflammation and lack of mucosal homeostasis.

In addition to the upregulation of PAFR, we observed enhanced epithelial repair after incubation of healing wounds with the pro-inflammatory lipid mediator PAF. PAF is an endogenous PAFR ligand. PAF administration has beneficial effects on keratinocyte wound healing, and therapeutic PAF antagonists delay oral and gastric mucosal ulcer healing[11, 34]. However, PAF delays wound closure in corneal cells[35] and PAFR antagonists have pro-repair effects on corneal injury and in rodent colitis models[9, 12]. These studies suggest that PAF effects are likely context dependent and may differ during acute inflammatory response to injury vs. persisting chronic inflammation. Further, we demonstrate that co-incubation of TNFα and IFNγ with PAF have a synergistic effect on wound healing, most likely due to the previously described crosstalk between TNFα and PAFR. We observed increased PAF levels two days after intestinal injury, which corresponds to a transition between pro-inflammatory and pro-resolution/repair phases in the mucosal wound healing model. Elevated mucosal levels of PAF and its receptor, as observed in individuals with IBD, might be a consequence of failed resolution of inflammation with associated impaired mucosal repair.

We observed PAF induced activation of EGFR, a receptor known to simultaneously activate multiple pathways and facilitate epithelial wound healing[36, 37]. Previous studies have implicated a central role for EGFR in GPCR signaling. Lemjabbar et al have described ADAM10-mediated EGFR transactivation by PAF induced PAFR signaling[17]. ADAM10 induces shedding of HB-EGF, an important EGFR ligand which is detected after injury in epithelial cells and increased after PAF treatment of model ovarian epithelial cells[16, 38, 39]. Tokumaru et al demonstrated shedding of HB-EGF after scratch wounding in keratinocytes, leading to enhanced migration and wound healing[40]. Activation of EGFR by HB-EGF results in a long-lasting robust phosphorylation at Y845 residue. Analogous ADAM10 activation as well as pEGFR-Y845 phosphorylation consistent with its activation was observed in our study. EGFR downstream signaling pathways that promote cell migration include Src phosphorylation leading to tyrosine phosphorylation and activation of FAK, which influences cytoskeletal reorganization and focal cell matrix adhesion turnover[36, 41]. In addition, Src itself has been implicated in GPCR-mediated ADAM activation and pEGFR-Y845 phosphorylation[24, 36]. Previous studies also demonstrated G-protein mediated Src kinase involvement in PAF-induced EGFR transactivation as well as activation of EGFR, Src, FAK and paxillin following PAF treatment in various cell systems[16, 42]. We show PAF treatment activates pSrc-Y416 as well as phosphorylation of FAK on Y861 (Src-dependent) and Y^{397} (autophosphorylation), both involved in GPCR and EGF-stimulated cell mobility[34]. Additionally, we observed that PAF treatment leads to activation of Rac1. Different signaling pathways downstream of PAFR can lead to such an increase of active Rac1. EGFR activation has been linked to small GTPase Rac1 activation through Src, leading to enhanced cell migration in IECs[43]. Rac1 regulates cell migration and can be activated by GPCR and Src-mediated signaling downstream of NADPH oxidase NOX1,

leading to ROS generation which exerts a positive influence on collective migration of the intestinal epithelium[21]. In our study, we observed Rac1 activation as well as increased levels of ROS following PAF treatment. Similarly, PAF stimulation of bovine neutrophil increases ROS production[44]. Additionally, we showed that PAF treatment increases focal adhesion size, thereby influencing cell-matrix adhesions[25].

Biologic therapy, consisting of TNFα monoclonal antibodies (e.g. Humira), has been shown to induce and maintain remission in a number of patients with moderate-to-severe IBD refractory to conventional immunosuppressive drugs. However, such biologic therapy has had treatment failures[45, 46]. The possibility that TNFα plays a role in mucosal repair and that a chronic blockage of this cytokine might prevent an appropriate healing response has not been extensively explored. In our studies, inhibition of pro-inflammatory TNFα signaling by administration of a functionally inhibitory anti-TNFα antibody suppressed PAFR upregulation during wound healing. In addition, we observed impaired wound healing after anti-TNFα antibody treatment that is likely mediated by impaired upregulation of GPCRs like PAFR that signal to promote wound healing. In support of our observations showing pro-repair properties of TNFα, Bradford et al. demonstrated that TNFα plays a beneficial role in enhancing Wnt/β-catenin signaling during ulcer healing in IBD. TNFα neutralization by antibodies induces mucosal healing but blockade of soluble TNF can negatively influence colitis[10]. Mucosal healing induced by anti-TNFα mAb is likely a secondary response to anti-inflammatory depletion of pathogenic effector cells. Given that the blockade of soluble TNFα (Etanercept) does not induce effector cell apoptosis it is possible that TNFα neutralization impairs IEC TNFα signaling and reduced TNFα available to IECs which could contribute to the negative clinical outcomes. Anti-TNFα mAb therapy may reduce stem IEC activation in patients with persistent mucosal inflammation. Thus, chronic inhibition of this cytokine might have detrimental effects on mucosal repair which is in part mediated by TNFα dependent up-regulation of G protein coupled receptors such as PAFR.

Taken together, our findings highlight a novel pro-repair mechanism that is mediated by the pro-inflammatory cytokine TNFα, PAFR and PAF which serve to orchestrate mucosal repair and restore mucosal homeostasis (Figure 7). The initial pro-inflammatory response sets the stage for pro-repair mechanisms that restore the epithelial barrier. Therapeutic strategies therefore need to take into consideration the time and context of chronic mucosal inflammation in order to accurately target key mediators involved in the disease process at the time of treatment.

Methods

Mice.

C57BL/6 were purchased from the Jackson Laboratory. *Ptafr*$^{-/-}$ mice on a C57BL/6 background were a gift of Professor Takao Shimizu (University of Tokyo).

Human colonic enteroids (colonoids).

Human colonoids were provided by Jason Spence (University of Michigan). 2D epithelial intestinal monolayers from 3D colonoids were generated as described by Saxena et al[47].

Cell lines and culture conditions.

Human intestinal epithelial cells (IECs) (SKCO15, T84) were grown as previously described[21]. After reaching a resistance >700 Ohms × cm^2 (~5 days), they were stimulated with TNFα (100 ng/ml) and/or IFNγ (100 ng/ml) for 24 hours.

IEC monolayer wounding in vitro.

Wound closure was assessed using a scratch wound assay as previously published[21]. See Supplementary Information for details.

In vivo wounding of colonic mucosa.

A high-resolution, miniaturized colonoscope system equipped with biopsy forceps (Karl Storz; Germany) was used to injure the colonic mucosa at 5 to 10 sites along the dorsal artery and, healing was quantified on day 1 and 3 post-injury.

In vivo antibody administration.

Mice were given i.p. injections of 250 μg of anti-TNFα monoclonal antibody (clone MP6-XT3, cat 16-7322-81, ThermoFisher Scientific) 6 hours before colonic biopsy wounding. Healing was quantified, and wounds were harvested. Rat IgG1 kappa isotype control (eBRG1 cat 14-4301-82, ThermoFisher Scientific) was administered as control.

Immunoblot and immunofluorescence.

For cell lysis, IEC monolayers were harvested in RIPA buffer. Immunofluorescence was performed as described previously[21].

Reagents.

The following antibodies were used: Calnexin (cat. C4731) Sigma (Darmstadt, Germany); FAK (cat. 610088) BD Biosciences (Franklin Lakes, NJ); pFAK (Y861) (cat. PS 1008) Calbiochem (Darmstadt, Germany); pFAK (Tyr397) (cat. 3283), Src (cat 2108), pSrc (Tyr416) (cat. 2101), EGFR (cat. 4267), pEGFR (Tyr845) (cat. 2231) Cell Signaling Technology (Danvers, MA). The hydrocyanine probe ROSstar 550, LI-COR Biosciences (cat. 926–20000). PAF (cat. 60900, Cayman Chemical, Ann Arbor, MI), PCA 4248 (cat. 0571, Tocris, Bristol, UK). PAF and PCA were used at a concentration of 10 μM. rhTNFα, rhIFNγ, rhIL-1β and rhIL-6 (cat. 300–01A, cat. 300–02, cat 200–01B and cat. 200–06; Peprotech, Rocky Hill, NJ) were used at a concentration of 100 ng/ml. fMLF (cat. F3506, Sigma, Darmstadt, Germany) was used at a concentration of 100 nM. Anti-TNFα, anti-IFNγ and Mouse IgG1 kappa Isotype Control (Mab1 cat 16-7348-81; NIB42 cat 16-7318-81 and P3.6.8.1 cat 14-4714-82, ThermoFisher Scientific) were used at a concentration of 10 μg/ml. p65 NF-κB and control siRNA were from Cell Signaling Technologies (cat 6261S and 6201) and used as suggested by the manufacturer. The NF-κB inhibitors Flavopiridol

hydrochloride (cat ab 141300, abcam) and bay11–7082 (cat 196870, millipore sigma) were uses at a concentration of 1μM and 10μM respectively.

Luciferase assay:

SKCO15 cells were plated in 48 wells plates and transfected with 0.2 mg/well of pGL4.32 vector containing a promoter with 5 copies of NF-κB response elements driving expression of the luciferase reporter gene and 0.04 mg/well pRL-TK (expressing Renilla luciferase; Promega) as a control for transfection efficiency. Reporter activity was determined using the Dual Luciferase Reporter Assay System (catE1910, Promega).

Human monocyte isolation and culture:

Human Peripheral blood monocytes were isolated using the EasySep™ Human Monocyte Isolation Kit (cat 19359, Stem Cell technologies). Monocytes were cultured in low adherence plates and treated with TNFα and IFNγ for 24 hours.

Cell death:

Cell death was evaluated using the Click-iT® TUNEL Alexa Fluor® 488 Imaging Assay (cat C10245, ThermoFisher Scientific).

qPCR.

Total RNA was isolated from SKCO15 cells, T84 cells, human colonoids or colonic wounds using the RNeasy kit (Qiagen). Primer sequences can be found in supplementary information

Lipidomic analysis of PAF levels.

25–30 punch biopsies (3 mm) of intact tissue or wounded colon from 3 animals on Day 1, 2 and 3 after wounding were analyzed for PAF levels at the Mass Spectrometry Lipidomics Core Facility, Dept. of Pharmacology, University of Colorado. The experiments were performed with 3 biological replicates.

Intracellular ROS generation.

Epithelial cells were treated with PAF or control media for the indicated times and incubated with 15 μM hydro-Cy3 for 30 minutes at 37°C. Epithelial cells were pretreated with NAC (20 mM) or PCA 4248 (10 μM) 30 minutes prior to PAF treatment. Quantification of fluorescence intensity of ROS was determined using ImageJ software.

Spinning disk assay.

Cell adhesion strength was measured using the spinning disk system as previously described[25, 48]. For details see Supplementary Information.

Focal adhesion staining.

For staining of FAs, cells cultured overnight on fibronectin-coated surfaces were rinsed and permeabilized in cytoskeleton-stabilizing buffer for 10 min, fixed in 3.7% formaldehyde for 5 min, blocked in 33% goat serum in PBS, and incubated with primary antibodies against

FA component Vinculin (cat. V284, Sigma) followed by AlexaFluor-labeled secondary antibody (cat. A-21422, Thermo Fisher Scientific).

Rac1 activation assay.

Confluent SKCO15 cell monolayers were grown in 100 mm tissue culture plates and grid wounds were generated to enrich for migrating and spreading cells. Rac1 activity was determined by pulldown using the RhoA/Rac1/Cdc42 Activation Assay Combo Biochem Kit (cat. BK030; Cytoskeleton, Inc.) according to the manufacturer's protocol.

In situ hybridization (ISH).

Partial murine cDNA fragments for *Ptfar* (Genbank NM_001081211.2 corresponding to bases 565 to 1372) were amplified via PCR, cloned into pZERO, and sequence verified. *Ptafr* riboprobes were made using the Roche Sp6/T7 DIG labelling system (cat. 11175025910, Roche, Basel, Switzerland) following the manufacturer's instructions. See Supplemental Information for details.

ADAM10 activity assay.

SKCO15 cells were plated sparse (60%) in 24-well tissue culture plates and treated with PAF or control media. ADAM10 activity was measured using the fluorimetric SensoLyte 520 ADAM10 Activity Assay Kit (cat. AS-72226; AnaSpec) according to the manufacturer's protocol.

Statistical analysis.

Statistical comparisons were performed by one- or two-way ANOVA with Bonferroni's multiple comparison or unpaired two-tailed Student's *t* test, as appropriate. A *p* value of less than 0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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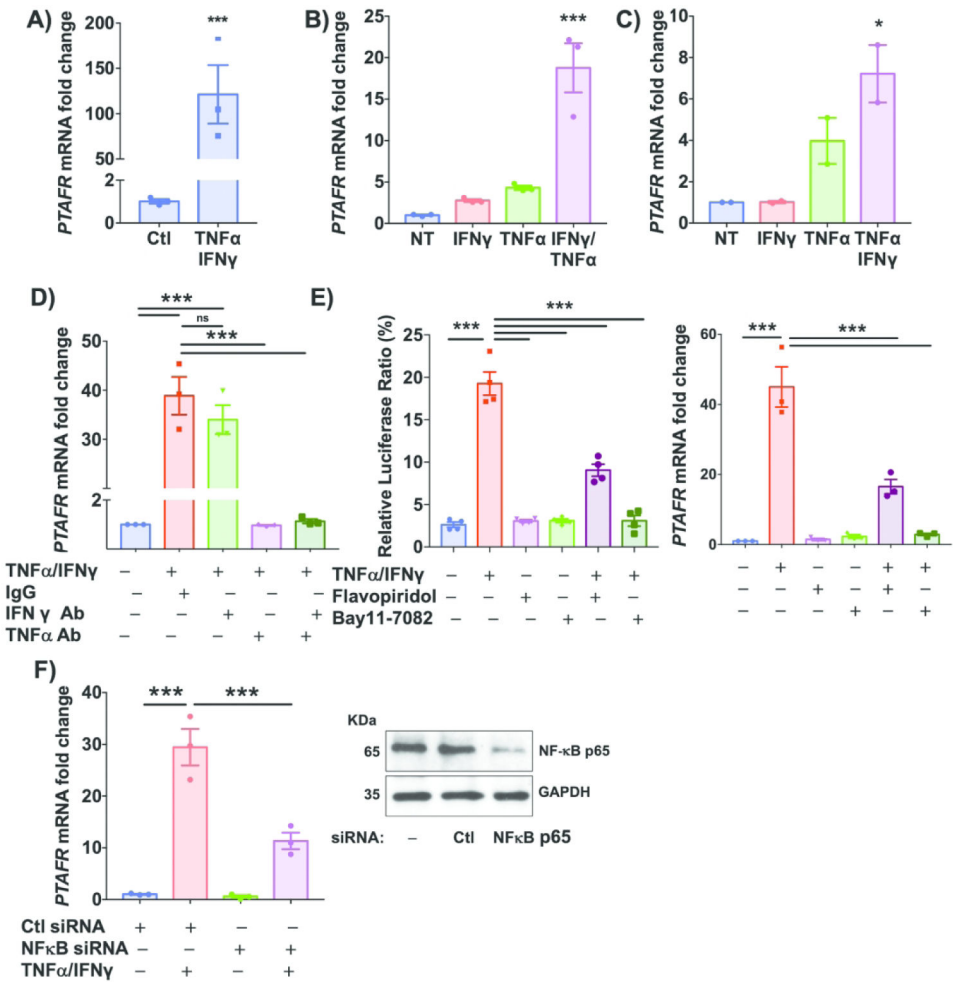

Figure 1. PAFR is synthesized through NF-κB signaling in response to TNFα

(A) SKCO15 model IECs treated with IFNγ (100 ng/ml) and TNFα (100 ng/ml). *PTAFR* mRNA levels were determined as a part of a GPCR PCR array (B) SKCO15 or (C) primary human colonoids were treated with IFNγ (100 ng/ml) and/or TNFα (100 ng/ml). *PTAFR* mRNA levels were determined by qPCR. (D) SKCO15 cells were pre-incubated 30 min with 10 μg/ml of IgG or neutralizing anti-TNFα, neutralizing anti-IFNγ and a mix of both antibodies; cells then were treated with IFNγ (100 ng/ml) and TNFα (100 ng/ml). *PTAFR* mRNA levels were determined by qPCR. (E) SKCO15 model IECs were pre-incubated 30 min with vehicle or 1μM Flavopiridol or 10μM Bay 11–7082 and then treated with IFNγ (100 ng/ml), TNFα (100 ng/ml). *PTAFR* mRNA levels were determined by qPCR. (F) NF-κB p65 was silenced in SKCO15 cells. After 24h, cells were treated with IFNγ (100 ng/ml) and TNFα (100 ng/ml) and *PTAFR* mRNA levels were determined by qPCR. Statistical comparisons performed using one-way ANOVA with Bonferroni's multiple comparison (***, $p < 0.001$; mean ± SEM); NT, vehicle control.

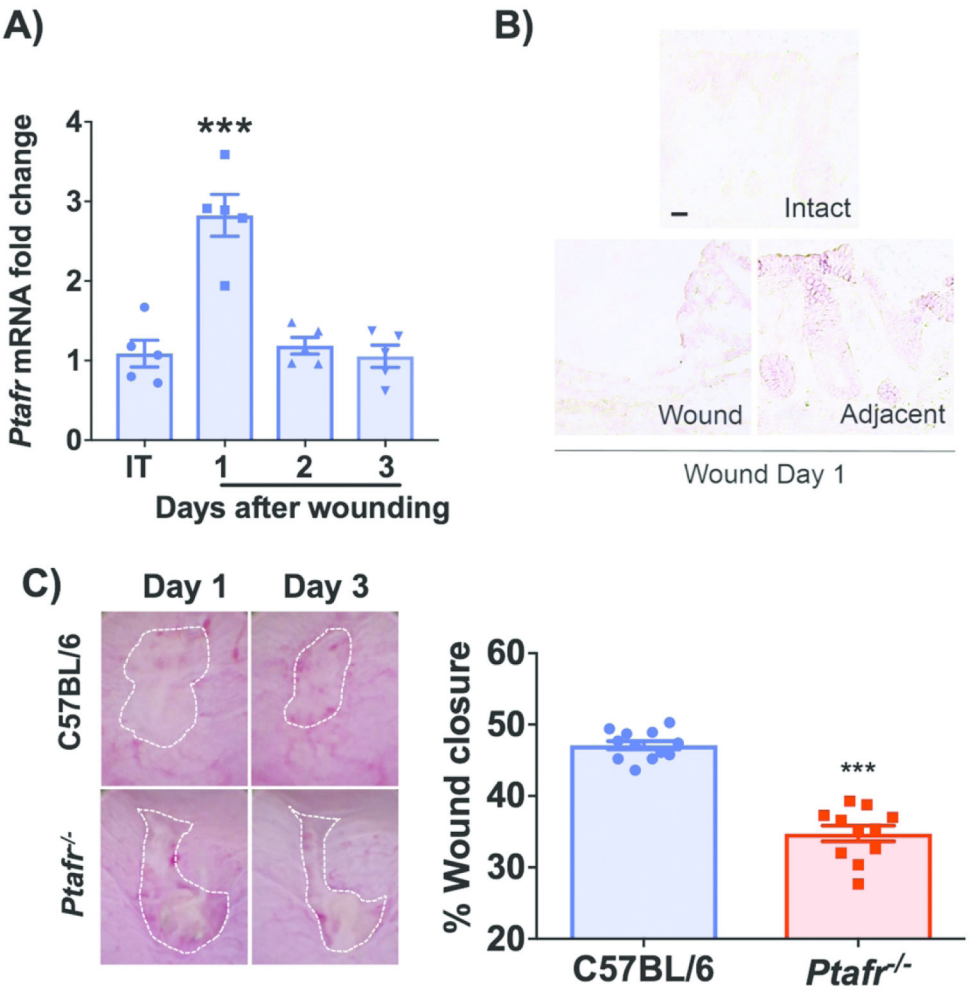

Figure 2. PAFR is important for *in vivo* intestinal mucosal wound repair.

(A) *Ptafr* mRNA levels in 3-mm punch biopsies of resealing colonic wounds on different days post injury compared to intact tissue analyzed by qPCR (n=8–10). (B) In-situ hybridization analysis of *Ptafr* expression in frozen sections from intact colon and 1 day after wounding (scale bar: 50 μm; original magnification 20×; representative images from 3 experiments). (C) Endoscopic images of colonic mucosal wounds in $Ptafr^{-/-}$ (n=11) compared with C57BL/6J mice (n=12) at days 1 and 3 post injury. Graph shows quantification of wound closure. Statistical comparisons performed using one-way ANOVA with Bonferroni's multiple comparison and unpaired two-tailed Student's t test with Welch's correction (***, $p < 0.001$; mean ± SEM). IT, intact tissue; NT, vehicle control.

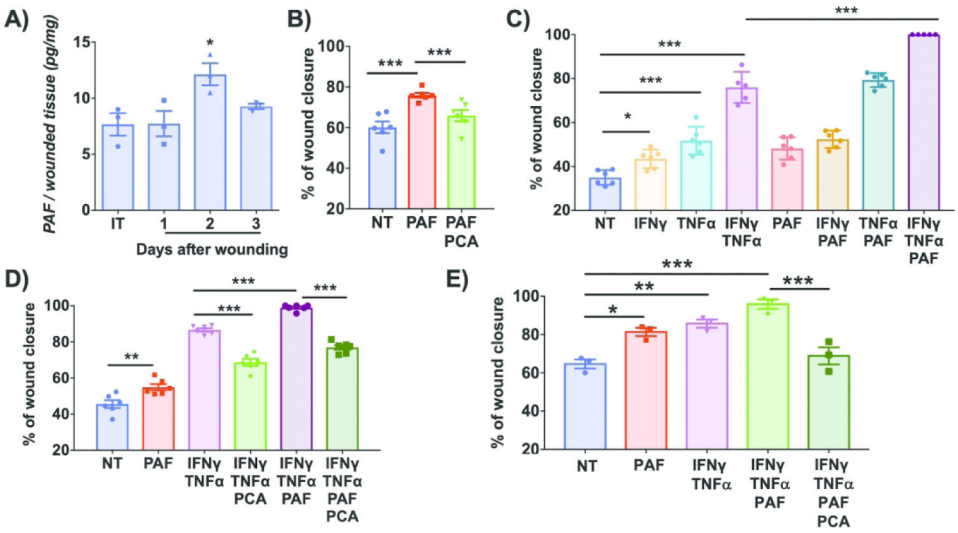

Figure 3. PAF is released in response to intestinal mucosal injury to promote repair.

(A) PAF levels in 3-mm punch biopsies of resealing colonic wounds on different post-injury days compared to intact tissue analyzed by lipodomics (n=3). (B, C, D) Wound areas of scratch wounded SKCO15 IEC monolayers, incubated with various treatments were continuously imaged. Percent wound closure was calculated by comparison of 0 and 18 or 24 hours post injury. Wounded IECs treated with (B) PAF (10 μM) with or without PAFR antagonist PCA 4248 (10 μM) for 24 hours; (C) IFNγ (100 ng/ml), TNFα (100 ng/ml), and PAF alone or in combination for 24 hours; (D) IFNγ, TNFα, and PCA 4248 alone or in combination for 18 hours. (E) Wounded primary IECs treated with PAF, IFNγ (100 ng/ml) and TNFα (100 ng/ml) and with or without PAFR antagonist PCA 4248 (10 μM) for 12 hours. Experiments were repeated 3 times, results of 1 representative experiment are shown. Statistical comparisons performed using one- or two way ANOVA with Bonferroni's multiple comparison (*, p< 0.5; **, p< 0.01; ***p< 0.001; mean ± SEM). IT, intact tissue; NT, vehicle control.

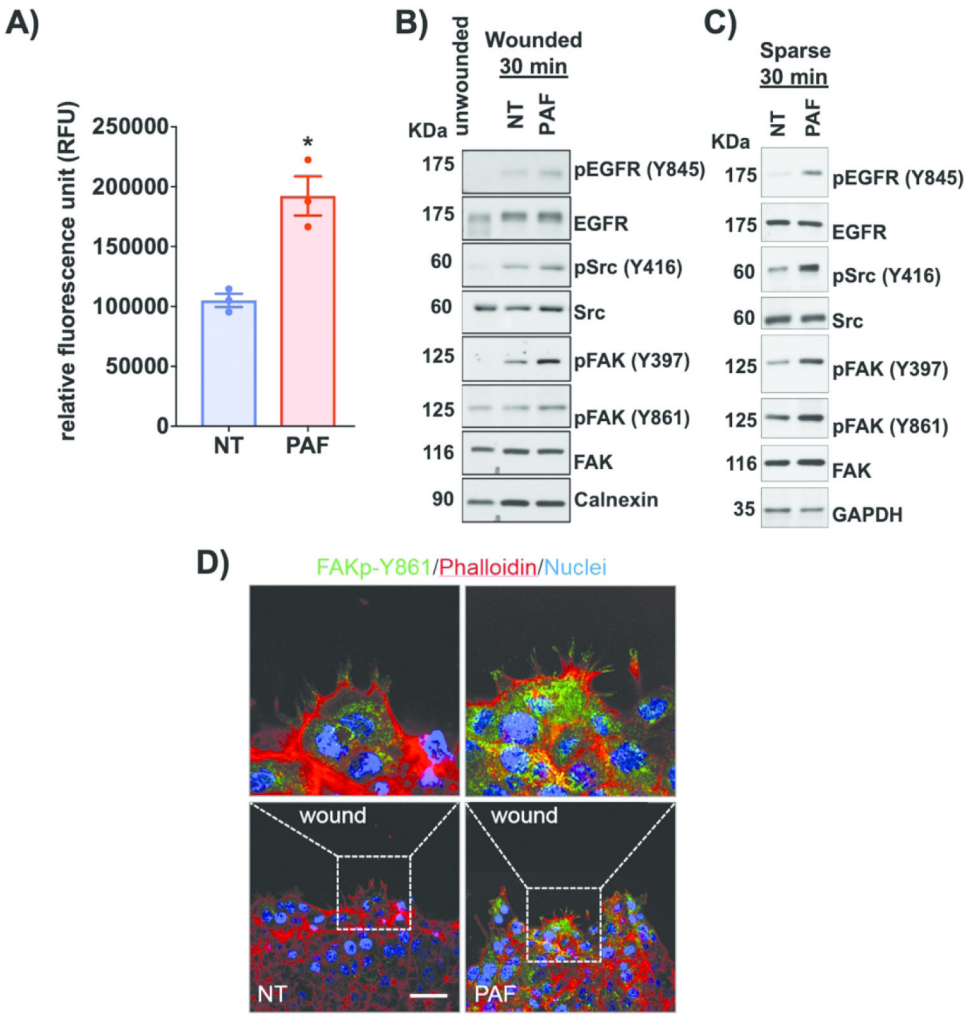

Figure 4. PAF promotes cell migration via ADAM10 mediated activation of EGFR signaling pathways.

(A) Fluorimetric ADAM10 activity assay performed on spreading SKCO15 model IECs treated with PAF (10 μM) or control at 30 min (*, $p < 0.5$; n=3; mean ± SEM). Immunoblotting was performed on lysates from scratch-wounded SKCO15 monolayers (B) and sparse primary IEC cultures (C) treated with PAF or control for 30 min. Levels of pEGFR, pSRC, and pFAK (Y397, Y861) were compared with total EGFR, SRC, FAK and Calnexin to assess activation (representative blots from 3 experiments). (C) Confocal micrographs of the focal contacts in migrating IECs at the leading edge of the wound after 2-hour treatment with control or PAF showing staining of pFAK (Y861), phalloidin (F-actin), and nuclei. (Scale bar 50 μm; original magnification 40×; representative images from 3 experiments). Statistical comparisons performed using unpaired two-tailed Student's *t* test with Welch's correction. NT, vehicle control.

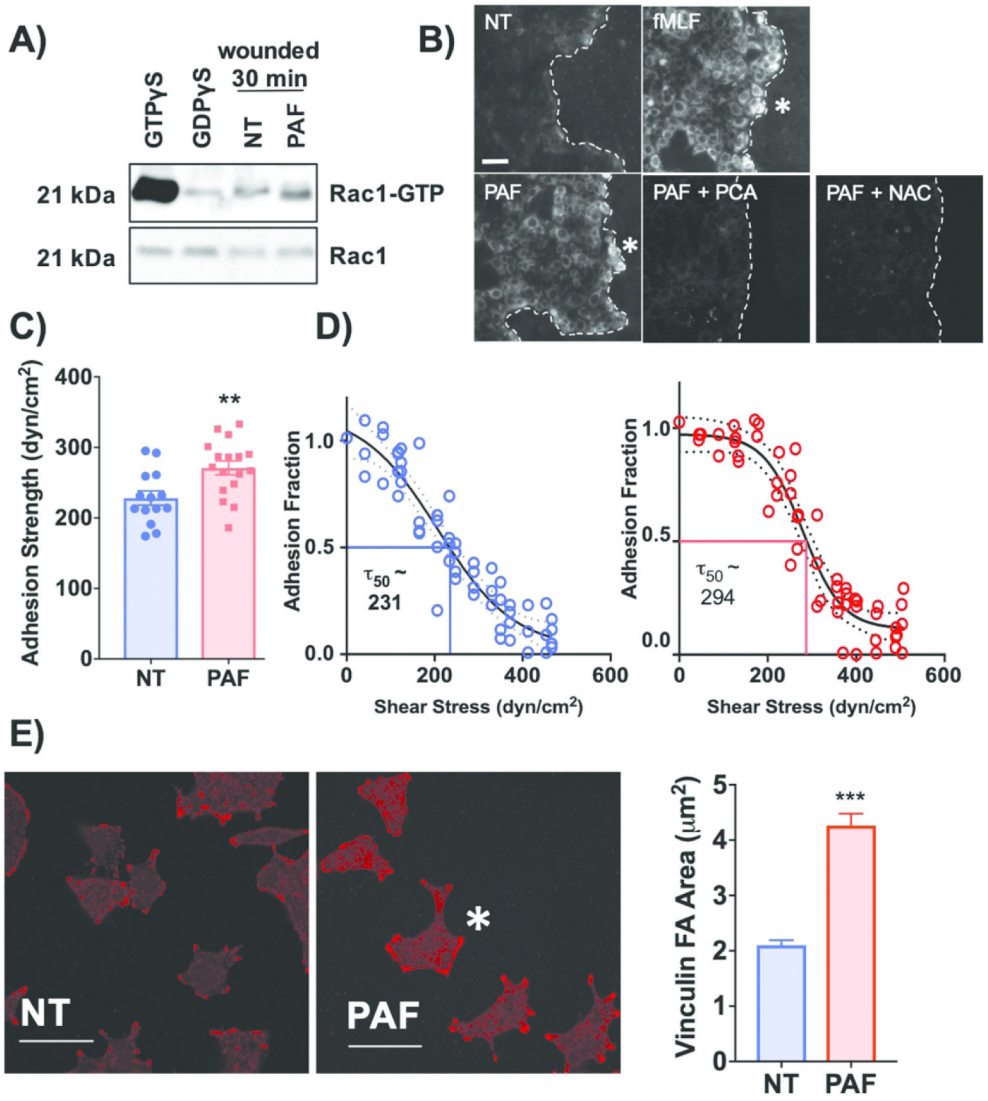

Figure 5. Rac1 activation, ROS generation, and increased cell-matrix adhesion amplified by PAF treatment.

(A) Treatment of scratch-wounded SKCO15 IECs with PAF (10 μM) or control for 30 min followed by analysis of Rac1 activation using a pulldown activation assay (representative blots from 3 experiments). (B) SKCO15 IECs were incubated with fMLF (100 nM), PAF (10 μM) alone and in combination with the ROS scavenger NAC (5 mM) or PCA 4248 (10 μM) for 15 min. ROS generation was detected by confocal microscopy using the fluorescent hydro-Cy3 dye in scratch-wounded monolayers adjacent to the wound edge, as indicated by asterisks (100 μm; original magnification 20×; representative images from 3 experiments). (C) Adhesion strength measurements of spreading SKCO15 IECs treated with PAF (1 μM) (n=17) or control (n=14) for 6 hours. (D) Representative adhesion profiles for each condition. τ_{50} is a metric for adhesion strength representing the shear stress at which 50% adherence is observed. (E) Vinculin staining performed on spreading SKCO15 IECs treated with PAF (1 μM) or control for 6 hours; asterisk indicating increased staining (50 μm; original magnification 60×). (F) Quantification of focal adhesion (FA) area (n=2). Statistical

comparisons were performed using unpaired two-tailed Student's *t* test with Welch's correction. (**, $p < 0.01$; ***, $p < 0.001$; mean ± SEM). NT, vehicle control.

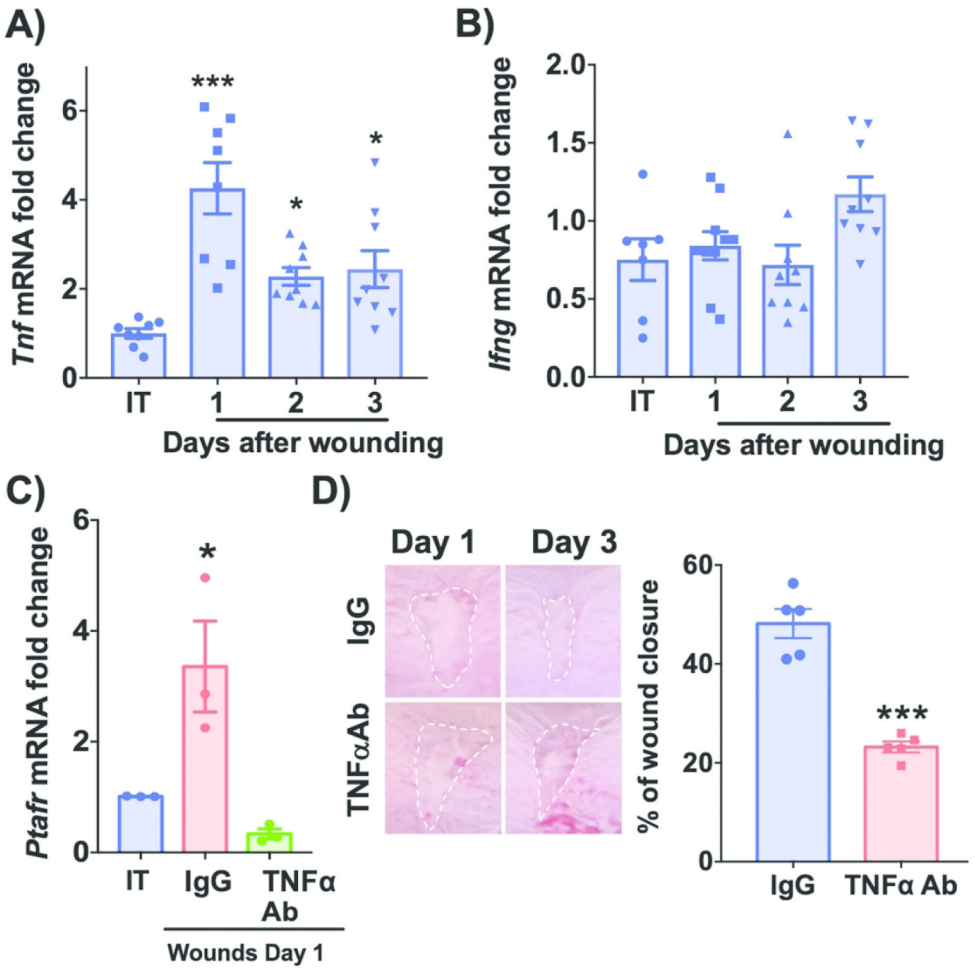

Figure 6. Inhibition of TNFα signaling *in vivo* ablates PAFR upregulation after injury and delays intestinal mucosal wound healing.

(A) *Tnfa* and (B) *Ifng* mRNA levels in 3-mm punch biopsies of resealing colonic wounds compared to intact tissue analyzed by qPCR (n=3). (C) *Ptafr* mRNA levels in 3-mm punch biopsies of resealing colonic wounds isolated from mice treated with a TNFα neutralizing antibody or control on different days post injury compared to intact tissue analyzed by qPCR (n=3). (D) Endoscopic images of colonic mucosal wounds 1 and 3 days after biopsy injury in C57BL/6J mice treated with a TNFα neutralizing antibody or IgG control. Graph shows quantification of wound closure (n=5). Statistical comparisons were performed using one-way ANOVA with Bonferroni's multiple comparison and unpaired two-tailed Student's *t* test with Welch's correction (*, $p< 0.5$; ***, $p< 0.001$; mean ± SEM). IT, intact tissue; Ctl, control.

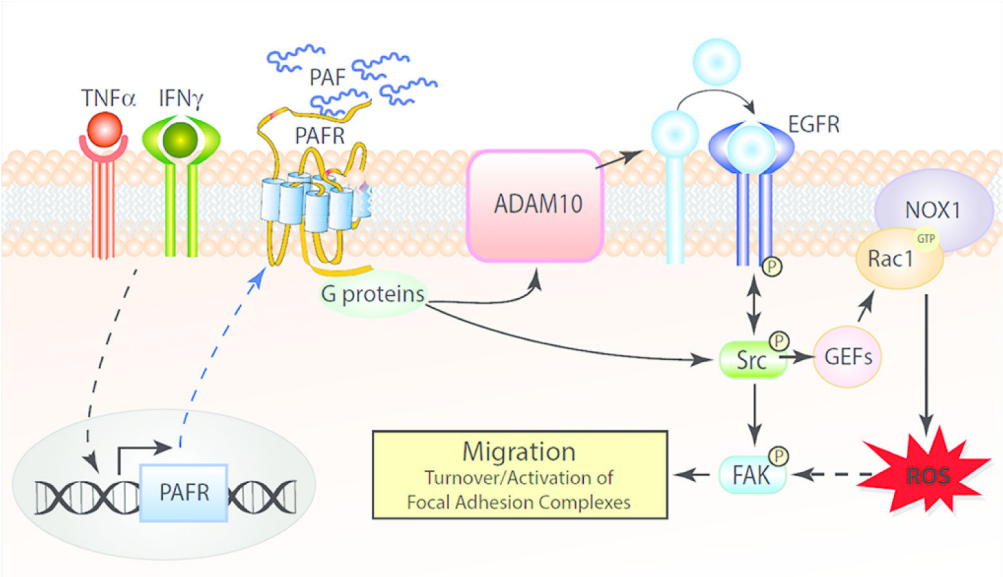

Figure 7. Schematic model illustrating the molecular mechanism of PAFR signaling during epithelial wound repair.

Our working hypothesis is that in response to wounding TNFα/IFNγ signaling increases expression of *PAFR*. Binding of PAF leads to PAFR stimulation which causes Src phosphorylation and ADAM10 activation, inducing cleavage of EGFR ligands, such as HB-EGF, and EGFR activation. Stimulation of EGFR further enhances phosphorylation of Src, which subsequently activates Rac1 via GEFs, increasing ROS accumulation and phosphorylation of FAK. In parallel, Src activation induces FAK phosphorylation to further promote turnover of focal adhesion complexes leading to modulation of cellular migration.