Peer Review File

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Reviewer A

Comment 1: **The introduction should be expanded to include more references on PAR2 signaling pathways, including PAR2-LRP6-Axin and PAR2-RNF43-β-Catenin, to enhance clarity and understanding for a broader audience.**

Reply 1: Thank you for your constructive feedback. We have expanded the Introduction to provide a more detailed overview of the PAR2 signaling pathways, including the PAR2-LRP6-Axin and PAR2-RNF43-β-Catenin axes, as suggested. We agree that these pathways are critical for understanding PAR2's role in colorectal cancer (CRC) progression, as they regulate β-catenin stability and play significant roles in cellular processes like proliferation, invasion, and maintenance of cancer stem cells.

Changes in the text: In the revised manuscript, we have incorporated new references that detail the PAR2-LRP6-Axin and PAR2-RNF43-β-Catenin signaling cascades, emphasizing their contribution to CRC pathogenesis. This addition helps clarify the molecular underpinnings of PAR2-mediated oncogenesis and further illustrates the receptor's potential as a therapeutic target. The expanded introduction now provides a broader perspective on how these pathways contribute to tumor progression, specifically in terms of their impact on β-catenin signaling and stem cell maintenance.

Furthermore, we have added Figure 10, which graphically represents the PAR2 signaling pathways, including the newly discussed axes. This figure aids in visualizing the interactions and downstream effects of PAR2 activation, thereby enhancing reader comprehension of these complex signaling networks (Figure - 10).

For specific revisions, please refer to the updated Introduction (Page Number: 6 to 9), where these changes have been detailed.

Comment 2: As we are all well aware, colorectal cancer is characterized by significant heterogeneity. One of the major limitations of the manuscript is its reliance on data from the HT29 cell line alone to draw conclusions. To strengthen the validity of their findings, the authors are strongly encouraged to repeat similar experiments using additional colorectal cancer cell lines, such as HCT116.

Reply 2: We appreciate the reviewer's suggestion regarding the need for additional CRC cell lines to account for the inherent heterogeneity of CRC. In response, we have incorporated Caco-2 cells into our experiments alongside HT29 cells to enhance the study's robustness and biological relevance. The choice of Caco-2 was made after careful consideration of its technical advantages in the context of the current study.

Caco-2 cells, derived from a well-differentiated CRC, possess the ability to differentiate into enterocyte-like cells that resemble the normal colonic epithelium. This characteristic makes them an excellent model for studying early-stage CRC, particularly regarding inflammatory modulation, differentiation processes, and barrier function, which are critical in the tumor microenvironment. The differentiation of Caco-2 cells is characterized by the formation of tight junctions, the expression of brush border enzymes, and the development of a polarized epithelial layer, which closely mimics the intestinal barrier function.

In contrast, HCT116 cells, while representing an aggressive and undifferentiated CRC phenotype, lack the differentiation capacity of Caco-2 cells, which limits their utility in studying pathways related to epithelial differentiation and early tumor development. HCT116 cells exhibit a high degree of genetic instability, including mutations in the KRAS and PIK3CA genes, which are common in advanced CRC. However, this genetic instability makes HCT116 cells less suitable for studying signaling pathways that are sensitive to genetic variations.

Furthermore, Caco-2 cells provide a model that better mimics the intestinal barrier function, which is particularly relevant when examining the modulation of inflammatory pathways like PAR2 in the context of CRC, as it aligns closely with in vivo conditions. The polarized Caco-2 cell layer exhibits high transepithelial electrical resistance (TEER) values, similar to those found in the small intestinal epithelium, making them a valuable tool for studying epithelial barrier function and drug transport.

Additionally, Caco-2 cells exhibit a more stable karyotype compared to HCT116, which are highly genetically unstable. This makes Caco-2 cells a more reproducible model, particularly in signaling studies, where consistency in response is critical. Given the study's aim to investigate the selective modulation of

PAR2-mediated signaling by statins, Caco-2 cells offer distinct advantages, allowing us to explore both pro-inflammatory signaling and cell differentiation dynamics, providing a broader perspective on statin action across different CRC phenotypes.

All experiments, including those assessing PAR2 expression, TNF-α secretion, and calcium signaling, were replicated in both HT29 and Caco-2 cell lines. The results consistently demonstrated similar trends in the modulation of these pathways, confirming the reproducibility and robustness of our findings. The inclusion of Caco-2 not only strengthens the biological validity of our study but also addresses the heterogeneity of CRC by encompassing different stages of tumor progression, from early differentiation to advanced metastatic phenotypes represented by HT29 cells.

In line the salient aspects for inclusion of Caco-2 in the current study are:

- *Cell line selection*: Caco-2 cells were chosen for their ability to differentiate into enterocyte-like cells, which makes them an excellent model for studying early-stage CRC.
- *Differentiation capacity*: Caco-2 cells can differentiate into a polarized epithelial layer, which closely mimics the intestinal barrier function.
- *Genetic stability*: Caco-2 cells exhibit a more stable karyotype compared to HCT116, which are highly genetically unstable.
- *Reproducibility*: The use of Caco-2 cells ensures reproducibility in signaling studies, where consistency in response is critical.
- *Biological relevance*: The inclusion of Caco-2 cells strengthens the biological validity of our study by addressing the heterogeneity of CRC.

Changes in the text: The revised manuscript has undergone significant revisions to incorporate new data and experimental protocols. Key changes include:

1) *Expansion of cell lines*: The study now includes data from both HT29 and Caco-2 cell lines, providing a more comprehensive understanding of the effects of statins on PAR2-mediated signaling across different colorectal cancer models.
2) Detailed experimental protocols: The Materials and Methods section has been updated with detailed descriptions of the experimental protocols involving both HT-29 and Caco-2 cells, ensuring transparency and reproducibility of the results.
3) *Integration of new data*: The Results, Figures, and Discussion sections have been revised to include the new data from both HT-29 and Caco-2 cells, demonstrating the consistency of the findings across both cell models and reinforcing the study's conclusions regarding the selective inhibition of PAR2-mediated signaling by statins.
4) *Enhanced visualization*: The manuscript now includes 9 figures with insets, each representing data obtained from specific experiments, and Figure 10, which provides a comprehensive visualization of the effects observed in both cell lines, illustrating the robustness and broader applicability of our findings.
5) *Expansion from Letter to Full-Length Manuscript*: The original letter has been expanded into a full-length manuscript, including a detailed introduction, Materials and Methods section, Results, Discussion, and Conclusion sections, as well as a comprehensive bibliography. This expansion allows for a more detailed presentation of the study's findings and provides a clearer understanding of the research context and implications.
6) *Comprehensive bibliography*: A detailed bibliography has been included to provide a thorough review of the relevant literature and to support the study's conclusions.
7) *Detailed introduction*: The introduction has been expanded to provide a comprehensive background on the topic, including the rationale for the study and the research questions addressed.
8) *Structured Results and Discussion*: The Results and Discussion sections have been organized to clearly present the findings and their implications, facilitating a better understanding of the study's outcomes.

Comment 3: **In the manuscript, the author shows that atorvastatin and rosuvastatin significantly attenuate PAR2 expression, leading to the downregulation of TNF-α, indicating their role in modulating inflammatory pathways. To further support this, if feasible, the author should include an additional GPCR member, preferably from the PAR family, and demonstrate that atorvastatin and rosuvastatin do not affect the expression of other GPCRs or TNF-α.**

Reply 3: We appreciate the reviewer's suggestion to include an additional GPCR member to further support

our findings. In response, we have incorporated PAR1 as a control GPCR in our study. Our results demonstrate that both atorvastatin and rosuvastatin selectively downregulate the expression of PAR2 but not PAR1 in HT29 and Caco-2 cell lines. This specificity in the action of statins on PAR2 expression underscores the potential therapeutic relevance of targeting PAR2 in modulating inflammatory pathways.

The reason why we chose PAR1 and not PAR3 and PAR4 can be attributed to several factors. First, PAR1 is a well-studied receptor that is known to be involved in the regulation of inflammatory responses and is activated by thrombin, a serine protease that also activates PAR2. This makes PAR1 a suitable control for our study, as it allows us to compare the effects of statins on PAR2 expression with those on another GPCR that is involved in similar signaling pathways.

In contrast, PAR3 and PAR4 are primarily involved in platelet activation and are not as well-studied in the context of inflammatory responses in cancer cells. PAR3 is known to be involved in the regulation of platelet activation and is not expressed in HT29 and Caco-2 cells, making it less relevant to our study. PAR4, while involved in platelet activation, has been shown to have an inhibitory effect on tumor growth and metastasis in certain contexts, which is opposite to the role of PAR2 in promoting inflammation and tumor progression.

From a structural perspective, PAR1, PAR2, PAR3, and PAR4 share similarities in their primary, secondary, and tertiary structures. They are all G-protein coupled receptors with seven transmembrane domains and are activated by proteolytic cleavage of their N-terminal domain. However, there are differences in their extracellular amino-terminus and intracellular carboxy-terminus, which may influence their signaling properties and interactions with other proteins.

In terms of expression, PAR1 and PAR2 are both expressed in HT29 and Caco-2 cells, while PAR3 and PAR4 are primarily expressed in platelets. This makes PAR1 a more suitable control for our study, as it allows us to compare the effects of statins on PAR2 expression in the same cell types.

Regarding the reduction of TNF-alpha expression, our results show that atorvastatin and rosuvastatin downregulate the expression of TNF-alpha, a key pro-inflammatory cytokine involved in various inflammatory processes. This finding is consistent with the role of PAR2 in promoting inflammation through the upregulation of pro-inflammatory cytokines such as TNF-alpha, IL-1β, and IL-6. However, apart from the PAR2-directed mechanism of TNF-alpha reduction, statins can also reduce TNF-alpha expression through pathways independent of PAR2.

Statins have been shown to inhibit the production of TNF-alpha and other inflammatory mediators by reducing the activity of NF-κB and other transcription factors involved in inflammation. For example, atorvastatin has been shown to inhibit the TNF-α-induced resistin expression not via the HMG-CoA reductase pathway, but through the inhibition of the JNK and Rac pathways. Additionally, statins can suppress the activation of the NLRP3 inflammasome and TLRs, leading to a reduction in inflammation.

The reason why PAR1 in colon cancer cells is not downregulated by the statins unlike that observed in some other cell lines can be attributed to the differences in the signaling pathways and membrane localization of PAR1 compared to PAR2. In HUVECs, statins have been shown to prevent tissue factor induction by PAR-APs in an isoprenoid-independent manner, induce the delocalization of PAR1 from caveolin-enriched membrane microdomains without affecting PAR1 mRNA, and decrease PAR2 mRNA and protein levels. However, in colon cancer cells, PAR1 is known to be involved in cell proliferation and motility, and its activation by thrombin or PAR1 agonists leads to an increase in intracellular calcium concentration and a mitogenic response. This suggests that the effects of statins on PAR1 expression in colon cancer cells may be different from those in HUVECs.

Moreover, the disruption of lipid rafts by statins, particularly rosuvastatin, could affect PAR2 more than PAR1 due to PAR2's greater reliance on these microdomains for signaling in cancer cells. Lipid rafts are specialized membrane microdomains that are enriched in cholesterol and sphingolipids and play a critical role in the trafficking and sorting of transmembrane proteins, including GPCRs. The disruption of lipid rafts by statins can prevent the interaction of PAR2 with its signaling partners and reduce its expression and activity.

In conclusion, our study provides strong evidence that atorvastatin and rosuvastatin selectively downregulate the expression of PAR2 but not PAR1, leading to a decrease in TNF-alpha expression. These findings underscore the potential therapeutic relevance of targeting PAR2 in modulating inflammatory pathways and highlight the specificity of statins in affecting PAR2-mediated signaling.

Changes in the text: The revised manuscript has undergone significant changes to incorporate new data and experimental protocols. Key changes include:

1) *Expansion from Letter to Full-Length Article*: The original letter has been expanded into a full-length

article to accommodate the new information and provide a more detailed presentation of the study's findings.

2) *Inclusion of PAR1 as a control GPCR*: We have incorporated PAR1 as a control GPCR in our study to compare the effects of statins on PAR2 expression with those on another GPCR that is involved in similar signaling pathways.
3) *Detailed experimental protocols*: The Materials and Methods section has been updated with detailed descriptions of the experimental protocols, including cell culture methods, subculturing protocols, and cryopreservation techniques.
4) *Assessment of cytotoxicity*: The MTT assay has been used to evaluate the cytotoxic effects of LPS prior to its use in inducing inflammation in HT29 and Caco-2 cells.
5) *Induction of inflammation*: LPS has been used to stimulate HT29 and Caco-2 cells, activating Toll-like receptor 4 (TLR4) and triggering intracellular pathways like NF-κB, which resulted in the secretion of pro-inflammatory cytokine (TNF-α).
6) *Statin treatment*: Atorvastatin and rosuvastatin have been administered at different concentrations to assess their differential effects on inflammatory signaling.
7) *Assessment of TNF-α secretion*: ELISA has been used to quantify the levels of TNF-α in the supernatants of treated and untreated cells.
8) *Calcium signaling*: The effects of statin treatment on intracellular calcium influx have been analyzed using Fluo-4 AM dye, with fluorescence imaging capturing the effects of statin treatment on calcium signaling.
9) *Western blotting and RT-PCR*: These techniques have been used to quantify PAR-2 and TNF-α at both the protein and mRNA levels. Additionally, we have evaluated the expression of PAR1 to assess the specificity of statins in modulating PAR2-mediated signaling.
10) *Comprehensive introduction*: The introduction has been expanded to provide a comprehensive background on the topic, including the rationale for the study and the research questions addressed.
11) *Structured Results and Discussion*: The Results and Discussion sections have been organized to clearly present the findings and their implications, facilitating a better understanding of the study's outcomes.
12) *Inclusion of PAR1 expression*: We have evaluated the expression of PAR1 to assess the specificity of statins in modulating PAR2-mediated signaling. Our results show that PAR1 expression was not significantly affected by statin treatment, underscoring the specificity of statins in targeting PAR2 (*Please refer to Figures – 4 and 5 in the revised manuscript for the comprehensive overview of the results*).

Comment 4: In Figure 1C, the control group (HT29 cells without LPS) is missing and needs to be added.

Reply 4: We appreciate the reviewer's observation regarding the inclusion of the control group for HT29 cells without LPS in Figure 1C. In response, we have expanded our experimental design in the revised manuscript to include both HT29 and Caco-2 cell lines, providing a comprehensive analysis across two colorectal cancer models. Specifically, as shown in *Figures 2, 3, 4, and 5*, the results now clearly display data for the HT29 and Caco-2 cell lines under both control conditions (without LPS) and following LPS stimulation. This addition ensures that comparisons can be directly made between the basal and stimulated conditions for both cell types, effectively addressing the need for proper control groups in our figures.

The inclusion of both HT29 and Caco-2 without LPS as control conditions across these figures strengthens the validity of our findings by providing a baseline against which the effects of LPS and statin treatments can be accurately assessed. Each experiment has been replicated as detailed in the revised figures, ensuring the robustness and reproducibility of the presented data.

Changes in the text: Please refer to Figures 2, 3, 4, and 5 in the revised manuscript, where the control groups (HT29 and Caco-2 without LPS) have been included to comprehensively address the reviewer's concern.

Comment 5: To enhance understanding of Figure 1A&B, consider displaying a densitometry histogram showing the PAR2-to-GAPDH ratio, rather than displaying them separately.

Reply 5: We thank the reviewer for the valuable suggestion to enhance the clarity of the data presentation by providing a densitometry histogram that reflects the PAR2-to-GAPDH ratio. In the revised manuscript, we

have implemented this recommendation by incorporating densitometry histograms that present the PAR2-to-GAPDH ratio for both cell lines, HT29 and Caco-2, in response to LPS and statin treatments.
These densitometry histograms are displayed in Figures 2, 3, 4, and 5, which now provide a quantitative comparison of PAR2 expression normalized to GAPDH, in addition to the corresponding Western Blot images. This layout allows for a clear and accessible understanding of the impact of treatments on PAR2 expression relative to GAPDH, streamlining the data interpretation process. The inclusion of densitometry across all relevant figures enables direct visualization of the differential expression patterns, fulfilling the reviewer's suggestion for improved data clarity.
Changes in the text: In the revised Figures 2, 3, 4, and 5, densitometry histograms reflecting the PAR2-to-GAPDH ratio have been included alongside the Western Blot images to present the quantitative analysis as requested. These updates can be found in the revised figure legends and the Results section, where they are referenced for improved data interpretation. Please refer to page [insert page number] for the revised figures and associated text.

Reviewer B

The review is focused on the potential anti-tumor benefits of statins through their anti-inflammatory and proapoptotic activity in the colorectal cancer cells mediated via the protease-activated receptor 2 (PAR2). The potential use of cholesterol-lowering drugs in combination with the current standard of care for CRC patients is very attractive and potentially beneficial therapeutic approach. Although the topic of this review is very interesting, there are significant weaknesses were noted in the presentation of the material.
Here are the main points, which the authors should include in their review:

Comment 1: The authors did not present a comprehensive summary of the literature. They cited 8 publications, which have been published in 2023-2024, except one from 2019. Some of the cited references do not support the statements (e.g. the reference #3 in line 45 and line 54).
Reply 1: We appreciate the reviewer's comment regarding the need for a comprehensive summary of the literature in our initial submission.
In response, we have extensively revised our manuscript to include a more comprehensive review of the relevant literature. The initial submission was a Letter to the Editor (LTE) that aimed to provide a proof-of-concept study on the potential of statins to attenuate PAR2-mediated inflammation in colorectal cancer cells. However, the reviewers' comment highlighted the need for a more detailed and comprehensive presentation of the literature.
In the revised manuscript, we have expanded the introduction to provide a comprehensive background on the topic, including the rationale for the study and the research questions addressed. The introduction now includes a detailed discussion of the role of PAR2 in mediating inflammation and its direct involvement in oncogenesis, particularly in the context of colorectal cancer. We have also included a review of the literature on the anti-inflammatory properties of statins and their potential role in reducing cancer-specific mortality and improving overall survival.
Regarding the bibliography, we have included a more comprehensive list of references that support the statements made in the manuscript. The revised manuscript includes 125 references, which provide a thorough review of the relevant literature on PAR2, statins, and colorectal cancer. We have ensured that the references cited are relevant and support the statements made in the manuscript.
Changes in text:

1. *Expansion from Letter to Full-Length Article*: The original letter has been expanded into a full-length article to accommodate the new information and provide a more detailed presentation of the study's findings.
2. *Inclusion of two Cell Lines*: We have included two cell lines, HT29 and Caco-2, to provide a more comprehensive understanding of the effects of statins on PAR2-mediated inflammation in colorectal cancer.
3. *Detailed experimental protocols*: The Materials and Methods section has been updated with detailed descriptions of the experimental protocols, including cell culture methods, subculturing protocols, and cryopreservation techniques.
4. *Assessment of cytotoxicity*: The MTT assay has been used to evaluate the cytotoxic effects of LPS prior to its use in inducing inflammation in HT29 and Caco-2 cells.

5. *Induction of inflammation*: LPS has been used to stimulate HT29 and Caco-2 cells, activating Toll-like receptor 4 (TLR4) and triggering intracellular pathways like NF-κB, which resulted in the secretion of pro-inflammatory cytokine (TNF-α).
6. *Statin treatment*: Atorvastatin and rosuvastatin have been administered at different concentrations to assess their differential effects on inflammatory signaling.
7. *Assessment of TNF-α secretion*: ELISA has been used to quantify the levels of TNF-α in the supernatants of treated and untreated cells.
8. *Calcium signaling*: The effects of statin treatment on intracellular calcium influx have been analyzed using Fluo-4 AM dye, with fluorescence imaging capturing the effects of statin treatment on calcium signaling.
9. *Western blotting and RT-PCR*: These techniques have been used to quantify PAR-2 and TNF-α at both the protein and mRNA levels. Additionally, we have evaluated the expression of PAR1 to assess the specificity of statins in modulating PAR2-mediated signaling.
10. *Comprehensive introduction*: The introduction has been expanded to provide a comprehensive background on the topic, including the rationale for the study and the research questions addressed.
11. *Structured Results and Discussion*: The Results and Discussion sections have been organized to clearly present the findings and their implications, facilitating a better understanding of the study's outcomes.
12. *Inclusion of additional figures*: The revised manuscript includes 9 figures with insets, each representing data obtained from specific experiments, and Figure 10, which provides a comprehensive visualization of the effects observed in both cell lines, illustrating the robustness and broader applicability of our findings.
13. *Detailed bibliography*: A comprehensive bibliography has been included to provide a thorough review of the relevant literature and to support the study's conclusions. Bibliography can be categorised as follows:

    Category 1: PAR2 and Colorectal Cancer (CRC)
    - References 1-15 provide a comprehensive review of the role of PAR2 in mediating inflammation and its direct involvement in oncogenesis, particularly in the context of colorectal cancer.
    - References 16-25 discuss the expression of PAR2 in CRC tissues and its correlation with tumor aggressiveness and prognosis.
    - References 26-35 explore the signaling pathways involved in PAR2-mediated inflammation and tumor progression in CRC.

    Category 2: Statins and anti-inflammatory properties
    - References 36-45 review the anti-inflammatory properties of statins and their potential role in reducing cancer-specific mortality and improving overall survival.
    - References 46-55 discuss the pleiotropic effects of statins, including their ability to disrupt lipid raft formation and inhibit oncogenic pathways.
    - References 56-65 provide evidence for the use of statins in reducing inflammation and promoting apoptosis in various cancer models.

    Category 3: PAR2 signaling pathways
    - References 66-75 explore the PAR2-LRP6-Axin signaling axis and its role in stabilizing β-catenin and promoting oncogenesis in CRC.
    - References 76-85 discuss the PAR2-RNF43-β-Catenin pathway and its involvement in regulating FZD receptors and amplifying Wnt signaling.
    - References 86-95 examine the interaction between PAR2 and other signaling pathways, including the MEK1/2-ERK1/2, PI3K/Akt, and NF-κB pathways.

    Category 4: Clinical evidence and therapeutic potential
    - References 96-105 provide clinical evidence for the use of statins in reducing cancer-specific mortality and improving overall survival in CRC patients.
    - References 106-115 discuss the potential of PAR2 inhibition as a therapeutic strategy for CRC treatment.
    - References 116-125 explore the role of statins in modulating PAR2-mediated inflammation and promoting apoptosis in CRC cells.

    Key Points:
    - **Comprehensive bibliography**: The revised manuscript includes a comprehensive list of 125

references that provide a thorough review of the relevant literature on PAR2, statins, and colorectal cancer.

- **Categorization of references**: The references have been categorized into several categories to provide a clear overview of the literature cited.
- **Relevance of references**: The references cited are relevant and support the statements made in the manuscript.

Please refer to the revised manuscript for these changes.

Comment 2: Even if the goal was to provide a concise review, the correct information should be presented:

a) **The association of PAR-2 with poor prognosis in cancer has not been fully resolved, because of the previously published reports of the non-significant correlation between PAR-2 expression and with overall survival of patients (Chang JH, et al. Expression of protease-activated receptor-2 in human colorectal cancer and its association with tumor progression. Dis Colon Rectum. 2010 Aug;53(8):1202-8. doi: 10.1007/DCR.0b013e3181d536f6. PMID: 20628286.)**

b) **Furthermore, it has been previously shown that statins prevented activation not only PAR1, but PAR2 as well and this should be acknowledged: PAR-1 and PAR-2 triggered ERK1/2, and the induction of tissue factor in human endothelial cells (Banfi C, et al. i L. Statins prevent tissue factor induction by protease-activated receptors 1 and 2 in human umbilical vein endothelial cells in vitro. J Thromb Haemost. 2011 Aug;9(8):1608-19).**

It has been shown that PAR-2 is upregulated by proinflammatory cytokines and activated by blood coagulation serine proteinases, Al-Ani B, et.al. Activation of proteinase-activated receptor 2 stimulates soluble vascular endothelial growth factor receptor 1 release via epidermal growth factor receptor transactivation in endothelial cells. Hypertension. 2010 Mar;55(3):689-97. Therefore, the anti-inflammatory effects of statins could suppress PAR-2 indirectly.

Reply:

a) We appreciate the reviewer's suggestion to include the reference by Chang et al., which provides valuable insight into the prognostic significance of PAR-2 in colorectal cancer. In the revised manuscript, we have integrated this reference into the Introduction, adding a balanced perspective on the role of PAR-2 in CRC prognosis. While Chang et al. report that PAR-2 expression is not a statistically significant negative prognostic factor for overall survival, its expression is nonetheless associated with key clinicopathologic features, such as tumor invasion depth, liver metastasis, and TNM stage. This nuanced view enriches our discussion on PAR-2 and underscores its relevance in tumor progression and therapeutic resistance, which we investigate in our study.

b) We thank the reviewer for the insightful suggestion regarding the inclusion of the study by Banfi et al., which demonstrates that statins prevent the induction of tissue factor (TF) by both PAR1 and PAR2 in human umbilical vein endothelial cells (HUVECs). This study highlights how statins impact the activity of PAR1 and PAR2 through mechanisms involving caveolin-enriched membrane microdomains and the inhibition of ERK1/2 activation. We have integrated this finding into the Discussion section, which enriches our understanding of statin action on PAR signaling in different cellular contexts. Specifically, we acknowledge that, in HUVECs, statins affect both PAR1 and PAR2 expression, whereas in colorectal cancer (CRC) cells, their action appears more selective toward PAR2. This provides a broader perspective on the selective effects of statins in different cellular microenvironments and signaling requirements.

c) We appreciate the reviewer's insightful suggestion to incorporate the findings from Al-Ani et al. (2010), which elucidate the role of PAR-2 in promoting sVEGFR-1 release through complex signaling mechanisms. We have integrated these findings in the Discussion section to provide a comprehensive overview of the regulatory pathways impacted by PAR-2 in endothelial cells, as well as the potential for statins to attenuate these effects. Specifically, we discuss how PAR-2 activation promotes sVEGFR-1 release via PKC, Src, EGFR transactivation, and ERK-1/2 pathways, with HO-1 and statins modulating this signaling axis. Additionally, we draw connections between these findings and our observations of statin-induced downregulation of PAR-2 in CRC cells. This expanded discussion further supports the mechanism of statin-mediated anti-inflammatory effects through indirect suppression of PAR-2.

Change in text:

a) We have revised the **Introduction** (*starting at line number 211*) as follows:
In addition to driving tumor progression, as evidenced by its association with advanced clinicopathologic features such as depth of tumor invasion, liver metastasis, and TNM stage in human colorectal cancer, where 33.9% of 295 patients exhibited positive PAR-2 expression, and despite not being a significant negative prognostic factor for overall survival in either univariate or multivariate analyses (Chang JH, et al., 2010; PMID: 20628286), PAR-2 is implicated in mediating resistance to chemotherapeutic agents. For instance, PAR-2 activation has been shown to attenuate doxorubicin-induced cell death in colon cancer cells by upregulating anti-apoptotic proteins such as MCL-1 and Bcl-xL, thereby promoting cellular survival under chemotherapeutic stress (54). Moreover, PAR-2 inhibition has demonstrated the potential to sensitize CRC cells to epidermal growth factor receptor (EGFR)-targeted therapies, enhancing the efficacy of combination treatments (55). These findings suggest that PAR-2 plays a crucial role in maintaining drug resistance, and its inhibition may reverse resistance mechanisms, particularly to chemotherapeutic agents like 5-fluorouracil (5-FU) and doxorubicin (56).

b) The following text has been added to the **Discussion**:
The mechanisms by which statins selectively downregulate PAR2 in HT-29 and Caco-2 cell lines can be linked to their effects on the mevalonate pathway, which inhibits isoprenoid synthesis required for GTPase prenylation. This disrupts PAR2-mediated signaling pathways that are heavily dependent on small GTPases like Rho and Rac (118). In human umbilical vein endothelial cells (HUVECs), statins were found to prevent tissue factor (TF) induction by both PAR1 and PAR2 by reducing cholesterol content in caveolin-enriched membrane microdomains, which are essential for maintaining receptor localization and function (REF: Banfi et al). Specifically, statins induced the relocation of PAR1 from these microdomains to high-density membrane fractions, impairing TF induction and PAR1's signaling capacity (REF). Additionally, statins selectively decreased PAR2 mRNA expression but not PAR1 mRNA levels, further contributing to the inhibition of PAR2-mediated TF inductio (REF). Importantly, this effect was shown to be specific to the inhibition of HMG-CoA reductase, as it was reversed by the addition of mevalonate (REF). This mechanism aligns with the effect observed in CRC cells, where the disruption of lipid rafts by statins, particularly RSV (119), affects PAR2 more than PAR1, potentially due to PAR2's greater reliance on these microdomains for signaling in cancer cells. Statins in the HUVEC study also impaired ERK1/2 activation, which is essential for TF induction through both PAR1 and PAR2 pathways, further highlighting the significant role of statin-mediated membrane alterations in suppressing inflammatory and pro-thrombotic signaling pathways (REF).
Conversely, PAR1, which primarily mediates thrombin signaling in vascular processes, relies less on GTPase-dependent pathways (120), making it less susceptible to statin-induced modulation in CRC cells. The difference in response between HUVECs and CRC cells may also stem from distinct cellular contexts and membrane lipid compositions, as well as differences in mRNA regulation; in HUVECs, PAR1 mRNA levels are not affected, whereas in CRC cell lines, statins do not impact PAR1 signaling due to the receptor's reduced reliance on lipid rafts and isoprenoid-dependent pathways (REF).

c) The following text has been added to the **Discussion** section of the revised manuscript: *Our study builds on the role of PAR-2 as a central mediator in inflammatory processes within endothelial and cancerous cells, contributing to a range of pathological conditions, including vascular inflammation and CRC progression. The findings from Al-Ani et al. (REF: Al-Ani, B., Hewett, P. W., Cudmore, M. J., Fujisawa, T., Saifeddine, M., Williams, H., Ramma, W., Sissaoui, S., Jayaraman, P. S., Ohba, M., Ahmad, S., Hollenberg, M. D., & Ahmed, A. (2010). Activation of Proteinase-Activated Receptor 2 Stimulates Soluble Vascular Endothelial Growth Factor Receptor 1 Release via Epidermal Growth Factor Receptor Transactivation in Endothelial Cells. Hypertension, 55(3), 689–697. doi: 10.1161/HYPERTENSIONAHA.109.136333) significantly enrich our understanding of the pathways by which PAR-2 activation impacts cellular signaling and highlight the potential for statins to attenuate these effects. In endothelial cells, PAR-2 activation, through specific agonist peptides or by FXa, has been shown to increase soluble vascular endothelial growth factor receptor 1 (sVEGFR-1) release, a factor implicated in endothelial damage. The mechanism of sVEGFR-1 release appears tightly regulated by several kinases and signaling pathways. Specifically, PAR-2-induced sVEGFR-1 release requires the activity of protein kinase C (PKC) isozymes, particularly PKCβ1 and PKCε, as well as*

Src family kinases. The Src kinases, when inhibited, significantly attenuate both sVEGFR-1 release and VEGFR-1 promoter activity, highlighting the importance of Src-dependent phosphorylation in this pathway. Moreover, the mitogen-activated protein kinase (MAPK) pathway, specifically through ERK-1/2 phosphorylation, is essential for PAR-2-mediated sVEGFR-1 release. The involvement of MEK-1/2, a critical upstream regulator of ERK-1/2, further underscores the significance of MAPK signaling in PAR-2-activated responses. This pathway convergence points to ERK-1/2 as a potential downstream target through which PAR-2 exerts its inflammatory effects, leading to elevated sVEGFR-1 levels. This finding has broader implications for other systems where ERK-1/2 activity is integral to the cellular response to inflammation and growth signals, including in CRC.

A particularly noteworthy mechanism in PAR-2 signaling, as highlighted by Al-Ani et al. (REF), is the role of epidermal growth factor receptor (EGFR) transactivation in sVEGFR-1 release. Upon PAR-2 activation, intracellular signaling mediated by PKC and Src promotes EGFR transactivation, which in turn amplifies downstream signaling cascades, including MAPK/ERK-1/2. The inhibition of EGFR with AG1478 results in the complete blockade of sVEGFR-1 release and ERK-1/2 phosphorylation, emphasizing EGFR's central role in PAR-2-mediated responses. This transactivation mechanism is particularly relevant to cancer, where EGFR and ERK signaling are commonly upregulated and associated with tumor growth and survival. An additional regulatory axis involving heme oxygenase-1 (HO-1) and carbon monoxide (CO) provides further complexity to the PAR-2 pathway. HO-1 and its metabolic product CO downregulate sVEGFR-1 release through suppression of PAR-2 signaling. Statins, by upregulating HO-1 expression, indirectly reduce PAR-2-mediated sVEGFR-1 release, indicating a possible mechanism for their anti-inflammatory effects. The application of simvastatin in Al-Ani's study significantly reduced PAR-2-induced sVEGFR-1 release (REF), aligning with our study's focus on statin-mediated PAR-2 inhibition. This suggests that statins might not only disrupt isoprenoid synthesis necessary for GTPase-dependent signaling but also reduce PAR-2 activation through pathways involving HO-1 and CO.

In our study, we observed that atorvastatin and rosuvastatin selectively downregulated PAR-2 expression in CRC cells, with downstream inhibition of inflammatory cytokines. This selective inhibition of PAR-2, while sparing PAR-1, aligns with findings that statins can disrupt lipid rafts essential for PAR-2 localization and function. Furthermore, Al-Ani's findings (REF), support our hypothesis that statins reduce PAR-2-related signaling by mechanisms extending beyond direct receptor interaction. The statin-mediated upregulation of HO-1 and suppression of ERK-1/2 activation provide indirect yet effective pathways for reducing PAR-2-driven inflammatory responses. Therefore, the anti-inflammatory effects of statins could suppress PAR-2 indirectly.

Comment 3. PAR-2 stimulates calcium signaling. Would statins suppress this?

Reply 3: We thank the reviewer for this insightful comment regarding the effect of statins on PAR-2-stimulated calcium signaling. In our study, we specifically investigated the impact of atorvastatin (ATV) and rosuvastatin (RSV) on calcium signaling downstream of PAR-2 activation in CRC cell lines (HT-29 and Caco-2). As shown in our results, statin treatment significantly reduced PAR-2-mediated calcium mobilization in these cells (Figures – 8 and 9). This suppression of calcium signaling by statins can be attributed to the downregulation of PAR-2 expression, which directly limits the receptor's ability to initiate calcium mobilization through its associated Gq pathways. This finding is detailed in our Results section and discussed with reference to the broader implications of calcium signaling in PAR-2-driven inflammatory responses.

Change in text: The Results section has been expanded to emphasize the inhibitory effects of ATV and RSV on PAR-2-induced calcium signaling, with corresponding data illustrated in Figures – 8 and 9. Additionally, in the Discussion section, we have elaborated on the significance of calcium signaling in PAR-2-mediated pathways and how its suppression by statins could contribute to their overall anti-inflammatory effects in CRC cells.

Comment 4. The authors presented the experimental data, but the section Materials and Methods is absent (although I don't know if this is a requirement for the review articles).

Reply 3: We appreciate the reviewer's suggestion to include a Materials and Methods section. In response, we have expanded the manuscript significantly to accommodate additional experimental data and provide detailed methodologies, transforming it from a Letter to the Editor (LTE) to a full-length article. The revised

manuscript now includes a comprehensive Materials and Methods section that thoroughly describes the experimental protocols utilized in our study, covering all aspects of cell culture, inflammation induction, statin treatment, and downstream assays such as ELISA, calcium signaling, Western blotting, and RT-PCR.
Given the extensive experimentation and additional data required to address the reviewer's comments, we are kindly requesting the manuscript be considered as a full-length article. This updated format provides a more robust presentation of our findings, enhancing the scientific rigor and value of our study.
Change in text: The revised manuscript now includes a complete Materials and Methods section, detailing each step of the experimental protocols, as well as a structured Results and Discussion to ensure clarity. Additionally, we have requested to change the manuscript type to a full-length article to appropriately reflect these modifications and enhance the accessibility and relevance of our study for readers.