Cytokine Expression in HepG2 Cells and the Effects of Budesonide

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Abstract: Inflammation has been shown to play a crucial role in regulating both the development of cancer and the response to therapy. Several cytokines are associated with inflammation, chronic illness, and subsequent cancer. Anti-inflammatory molecules show promise as a preventative therapy for high-risk populations by targeting inflammation. Glucocorticoids are widely recognized for their potent anti-inflammatory properties, but their therapeutic utility is often associated by the development of substantial adverse effects with prolonged administration. Budesonide, a synthetic glucocorticoid, offers a more favorable therapeutic index. Characterized by reduced systemic bioavailability compared to other glucocorticoids, budesonide demonstrates reduced classic glucocorticoid-induced side effects. Budesonide has been successfully employed in the management of inflammatory conditions such as ulcerative colitis, autoimmune hepatitis, and asthma. This therapeutic profile positions budesonide as a promising candidate for further exploration in various disease states characterized by chronic inflammation. To utilize the anti-inflammatory potential of budesonide, we aimed to determine its suitability as a prophylactic or therapeutic strategy for liver cancer, specifically hepatocellular carcinoma, by investigating its inflammation-modulating effects. We treated liver cancer cells (HepG2) with Lipopolysaccharide (LPS) that induced inflammatory cytokines like tumor necrosis factor (TNF) α and examined the effect of budesonide to suppress the cytokine expression. Our study provides novel insights into the LPS-induced inflammatory pathway in HepG2 cells and assesses the therapeutic potential of budesonide.

Keywords: inflammation, cancer, budesonide

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Introduction

Rudolf Virchow first proposed the correlation between inflammation and cancer in the mid-19th century. He noted that cancer often originates in areas of chronic inflammation and observed abundant inflammatory cells in tumor biopsies [1]. Since then, research has shown that inflammation is closely linked to all stages of cancer development and progression and the effectiveness of anti-cancer treatments [2,3,4,5]. Today, cancer-related inflammation is recognized as one of the hallmarks of cancer, with chronic inflammation being linked to an increased risk of malignancies and the progression of cancer in various types [6,7]. Given this relationship, harnessing inflammation [8] has become an important strategy for improving anti-cancer therapies. Clinical studies have highlighted the potent effects of non-steroidal anti-inflammatory drugs (NSAIDs), notably aspirin, in mitigating cancer risk [9]. High doses and long-term treatment with NSAIDs, however, lead to peptic ulcers, kidney diseases, and stroke [10,11].

During chronic inflammation, when the immune system is over-stimulated for long periods of time, it is generally treated with corticosteroids [12]. These medications suppress the immune system but can only be prescribed for a short time as they have serious side effects like weight gain, bone density loss, high blood pressure, etc. [13]. On the other hand, budesonide, a synthetic steroid of the glucocorticoid family has a high topical anti-inflammatory activity. Budesonide has been used orally for several immune-mediated gastrointestinal and liver diseases and as a nasal spray or by inhalation for allergic rhinitis, asthma, and chronic obstructive lung disease [14,15,16,17]. Budesonide has a high first-pass elimination by the liver (90%) with minimal systemic absorption and is therefore felt to cause fewer side effects than traditional glucocorticoids and to be generally well tolerated. Low systemic exposure to oral budesonide enables the use of budesonide as a prophylactic. Another advantage is that budesonide does not need dose tapering before discontinuation as it does not markedly affect endogenous cortisol production [14].

We selected budesonide for our study because the student who initiated the research has ulcerative colitis and uses budesonide to manage his inflammation. While a few studies suggest that budesonide may have potential for treating moderate to severe colitis [18] or preventing the progression of partially solid lung nodules [19], this study aimed to examine its effect on suppressing inflammation in HepG2 cells derived from a 15-year-old hepatocellular carcinoma patient.

In healthy individuals, the liver is constantly exposed to inflammatory stimuli from dietary sources and commensal bacterial products. It responds by producing acute phase proteins, complement components, cytokines, and chemokines and serves as a reservoir for various resident immune cell populations. [20, 21]. Traditionally viewed as a primarily metabolic and detoxification organ, we now understand the liver as a complex immune organ. Hepatocellular carcinoma (HCC), which accounts for 90% of liver cancer cases, is associated with chronic inflammation and fibrosis, often stemming from factors such as hepatitis B and C, along with alcoholic and nonalcoholic fatty liver diseases [22].

To mimic the inflammatory conditions prevalent in the liver, HepG2 cells were subjected to LPS stimulation, a well-characterized model of hepatic inflammation [23, 24]. HepG2 cells are known to secrete pro-inflammatory cytokines like TNF-alpha and IL-6 upon LPS stimulation [25] or hyperglycemic challenges [26]. HepG2 cells were selected as a suitable model system. By employing this experimental model, we sought to elucidate the therapeutic efficacy of budesonide in mitigating the inflammatory response characteristic of liver disease, with a specific focus on its impact on hepatocellular carcinoma.

Methods

Cell Culture

The human hepatocellular carcinoma cell line HepG2 was obtained from the American Type Culture Collection (ATCC, Maryland, USA). HepG2 cells were routinely grown in Dulbecco's Minimal Eagle Media (DMEM) media (Thermo Fisher) supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 mg/ml) (Thermo Fisher). Cells were grown at 37°C in disposable T-75 & T-25 flasks in a humidified atmosphere of 5% CO_2/95% air. The cells were split every three days in a ratio of 1:4.

Treatments

Novus Biological lipopolysaccharide (LPS) from *E.coli* was procured from Fisher Scientific and dissolved in media at a concentration of 1mg/ml. Budesonide (97%) was purchased from VWR and dissolved in DMSO at a concentration of 100 mg/ml.

CCK-8 Cell Viability Assays

To comprehensively evaluate the effects of LPS and budesonide on cell viability, HepG2 cells were exposed range of concentrations of each compound independently, as well as in combination with both. The cell viability was tested by incubating the cells with Dojindo molecular technologies CCK-8 WST-8 reagent [27]. WST-8 is reduced by dehydrogenases in cells to give an orange-colored product (formazan), which is soluble in the tissue culture medium. The amount of the formazan dye generated by dehydrogenases in cells is directly proportional to the number of living cells. The absorbance of cells was read at 450nm on an ELISA plate reader from Biorad.

Cytokine Expression

To systematically characterize the cytokine response elicited by LPS and the subsequent modulatory effects of budesonide, we carried out a proteomic profiling approach utilizing Abcam's human cytokine antibody arrays (ab133997). The Abcam cytokine array analyzes 42 human cytokines simultaneously, and cytokine arrays function similarly to ELISA assays but utilize a membrane substrate instead of a plate. Capture antibodies are arranged in spots on the membrane, with each spot pair representing a different analyte/cytokine. Conditioned media that would contain the cytokines secreted by the cells under the four treatment conditions were collected, added to the membranes, and incubated overnight. The membranes were washed and incubated with paired biotinylated detector antibodies provided in the kit, and finally, they were incubated with streptavidin HRP. The blots were exposed to a chemiluminescent substrate provided with the arrays from Abcam. The blots were exposed to GE Amersham Imager 680.

Analysis Using Image J Software

Densitometric analysis was carried out with ImageJ open-source software (http://rsbweb.nih.gov/ij). ImageJ was chosen for its capability to manipulate contrast settings without distorting true density, setting it apart from other software. The scanned images were inverted, and background signals were excluded from each reading based on the presence of three intentionally blank spots printed on the membranes.

Mathematical Formulation

Excel (Microsoft) was used for mathematical calculations. The formula used for analysis was: $X(Ny) = X(y) * P1 / P(y)$ where: X(Ny) represents the spot "X" density on array "Y." X(y) is the signal density at the spot of interest. P1 signifies the mean signal density on the "+ ref" reference array. P(y) stands for the mean signal density of the positive control on array "Y." This methodology allowed for the semi-quantitative assessment of cytokine levels in the samples, with a focus on accuracy and consistent data analysis using specialized software and mathematical formulas.

Confirming Cytokine Array Results with Gene Expression Analysis

RNA extraction from the HepG2 cells was done using the phenol-free RNA extraction kit (VWR). RNA was quantified with a nanodrop spectrophotometer from Thermo Fisher. Cytokine expression levels were quantified by examining RNA expression through reverse transcriptase polymerase chain reaction (RT-PCR). For reverse transcription, 2 µg of RNA was used. ProtoScript II Reverse Transcriptase kit from New England Biolabs was used for cDNA synthesis. Real-time PCR was performed using a powertrack SYBR master mix from Thermo Fisher.

Pre-designed primers for gene expression analysis were obtained from Integrated DNA Technologies (IDT). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as the housekeeping gene for normalization. The PCR was run on Thermo Fisher's Quantstudio-3 real-time PCR machine. The cytokines that were analyzed were TNF-α, Tumor growth factor (TGF)-beta, Interferon gamma (IFN-γ), IL-15, Oncostatin (OSM-1), IL-2, and Granulocyte-macrophage colony-stimulating factor (GMCSF). PCR reactions for each sample with all the primers in every experiment were done in triplicate, and the fold changes were calculated using the Delta-delta ct method ($2^{-\Delta\Delta Ct}$) [28].

Results and Discussion:

LPS and Budesonide Not Toxic to Cells

To comprehensively evaluate the interactive effects of LPS and budesonide, a comprehensive experimental design was implemented. Cells were subjected to a matrix of treatment conditions, including both individual and combinations of LPS and budesonide concentrations within the specified ranges. HepG2 cells were incubated for 24 hrs. in the presence of varying concentrations of LPS -1 µg/ml to 40 µg/ml and budesonide - 10^{-3} M to 10^{-10} M. None of the tested concentrations affected cell viability, as indicated by the CCK-8 assay (results not shown). Replicated experiments demonstrated consistent outcomes. Based on these results and established literature, LPS and budesonide concentrations of 4 µg/ml and 10^{-7} M, respectively, were selected for subsequent investigations. The experimental design aimed to determine if a 24-hour LPS exposure would induce inflammatory cytokine production in HepG2 cells and whether a 10^{-7}M budesonide treatment could attenuate this response.

Minimal or No Cytokine Induction by LPS and Inhibition by Budesonide

Conditioned media from HepG2 cells which were untreated, treated with 4 µg/ml LPS, treated with 10^{-7} M budesonide alone or with LPS and budesonide for 24 hrs., was collected and used to probe Abcam cytokine antibody arrays. Figure 1 shows the images of the Abcam cytokine arrays obtained from the GE Amersham Imager 680 scanner.

In contrast to previous literature, our findings revealed an unexpected absence of detectable levels of several LPS-induced cytokines, including TNF-α, IL-1 beta, IFN-γ, and IL-6, as assessed by cytokine array (Figure 1). This discrepancy between our results and prior studies warrants further investigation.

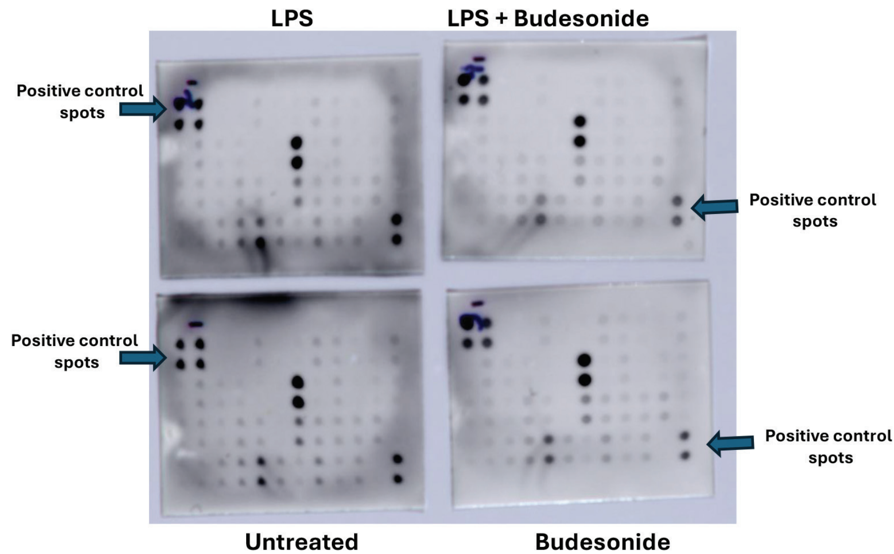

Fig.1: Abcam arrays were developed using a chemiluminescent substrate and exposed to GE Amersham imager 680 scanner to generate the images.

Given the absence of visually apparent cytokine expression differences, a quantitative analysis was performed. To accurately quantify cytokine levels, ImageJ densitometry software was employed to measure the signal intensities associated with antigen-specific antibody spots on the array. A fundamental principle underlying this analysis is the direct proportionality between signal intensity and antigen concentration within the sample. Relative differences in cytokine expression levels among treatment groups were determined by comparing signal intensities of individual antigen-specific antibody spots to the untreated control. By meticulously quantifying the signal intensity of each spot, we obtained a numerical representation of the corresponding cytokine abundance. This quantitative approach enabled a precise comparison of cytokine expression levels across different experimental conditions, facilitating the identification of subtle variations that might have been overlooked through visual inspection alone. Fold changes were calculated by comparing the signal intensities for each cytokine after background subtraction and normalization, as mentioned in materials and methods, such as mathematical formulation. Table 1: Lists the cytokines that show more than a 2-fold change in expression in LPS-treated cells when compared with untreated cells (row 1) and the cytokines that were suppressed by budesonide and LPS treatment when compared with LPS treatment alone (row 2).

Table 1: Shows the cytokines that were induced by LPS treatment and suppressed by Budesonide treatment. Fold changes after the normalization of the data are shown.

Cytokine	LPS treated/Untreated	LPS+budesonide/ LPS treated
IL-2	2.74	0.34
IL-1 alpha	2.50	0.50
IFN-γ	2.60	0.90
IL-15	5.60	0.20
GMCSF	2.01	0.58
IL-3	2.35	0.82
I-309	2.74	0.25

IL-1 alpha, IL-2, IL-3, IL-15, IFN-γ, GMCSF and I-309 which is small glycoprotein that belongs to the chemokine family showed fold change in expression above 2-fold though the induction of these cytokines were not visible on the arrays as the signal intensities were low. The induction of these cytokines was also suppressed by co-treatment of cells with LPS and budesonide.

Effect of Different Concentration of LPS on Cytokine Expression at the RNA Level

HepG2 cells were treated with different concentrations (2-20µg/ml) of LPS for 24 hours and analyzed for the expression of cytokines. Since the sensitivity of the Abcam arrays is low, we analyzed the cytokine expression at RNA level by RT-PCR. A comprehensive analysis of cytokine expression profiles was undertaken, specifically, the expression levels of TNF-α, IFN-γ, and OSM-1 (a member of the IL-6 family) as described in previous literature [24], as well as IL-15, GM-CSF, and IL-2, which exhibited significant induction in our cytokine array analysis. This focused study of a targeted cytokine panel allowed for a deeper understanding of the complex inflammatory response elicited by LPS and the potential modulatory effects of budesonide.

Figure 2 shows that 2-4µg/ml of LPS did induce the expression of Oncostatin (IL-6 family), IFN-γ, IL-2, and IL-15. Though at higher concentrations of LPS (8-20µg/ml) there was suppression in the expression of the cytokines as has been previously reported [29]. Cytokines GM-CSF and TNF-α showed increased expression at 2µg/ml LPS concentration and gave variable levels of expression at higher concentrations. TGF beta was not induced by LPS. Further experiments to analyze the suppression of cytokine expression were done using 4 µg/ml of LPS to induce cytokine expression and 10^{-7} M of budesonide to examine its effect on suppressing the induced cytokines [30].

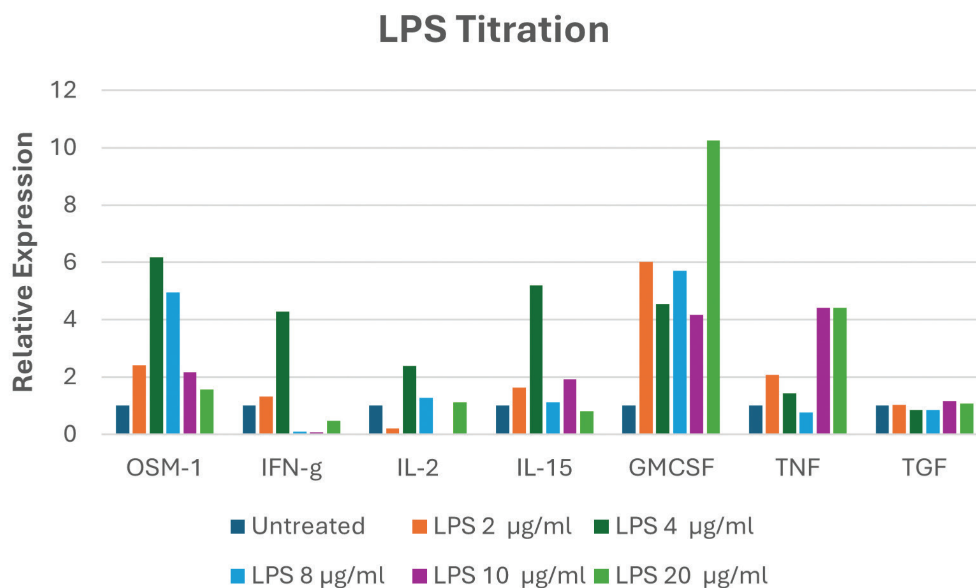

Fig. 2: Shows the induction of Oncostatin (IL-6 family), TNF-α IFN-γ, IL-2, IL-15, GM-CSF and TGF beta in HepG2 cells treated with LPS concentrations ranging from 2µg-20µg for 24 hours. The Y axis represents the expression level of the cytokines calculated by the Delta-delta ct method ($2^{-\Delta\Delta Ct}$).

LPS and Budesonide Impact Cytokine Expression

HepG2 cells treated with 4 µg/ml of LPS consistently showed a 6.86-fold increase in OSM-1 (a member of the IL-6 family), a 12.38-fold increase in IFN-γ, and a 4.23-fold increase in IL-15 levels (Figure 3). These results are averaged from five independent experiments. A paired t-test was employed to compare cytokine levels before and after treatment across all five experiments, with significance determined at $p<0.05$. Significant increases were observed for OSM-1 and IFN-γ expression, with p-values less than 0.05, as indicated by the asterisks in Figure 3. However, the p-values for IL-15 and GM-CSF were 0.08 and 0.6, respectively, indicating variability in their expression levels. TNF-α, TGF-beta, and IL-2 did not show consistent induction following LPS treatment and are therefore not included in Figure 3.

Treatment with 10^{-7} M budesonide alone did not induce the expression of IFN-γ, IL-15, or GM-CSF. However, budesonide treatment did result in an increase in OSM-1 expression (p=0.02). In our experiments, co-treatment with LPS and budesonide did not consistently suppress the induced cytokines OSM-1, IFN-γ, IL-15, and GM-CSF. Contrary to expectations, budesonide, in combination with LPS, led to an increase in IFN-γ (p=0.01) and GM-CSF expression, while IL-15 expression remained unchanged. This outcome differed from results observed in cytokine arrays, where budesonide and LPS co-treatment inhibited the expression of these cytokines. As we did not conduct a titration experiment with varying concentrations of budesonide and LPS, we cannot confirm whether a different concentration of budesonide might have effectively suppressed the cytokines analyzed in our study. We chose the 10^{-7} M concentration because previous studies reported that this concentration suppressed cytokine expression in colon cancer cells co-cultured with peripheral blood mononuclear cells (PBMCs) or in PBMCs stimulated with LPS [30], as well as in human lung epithelial cells [31].

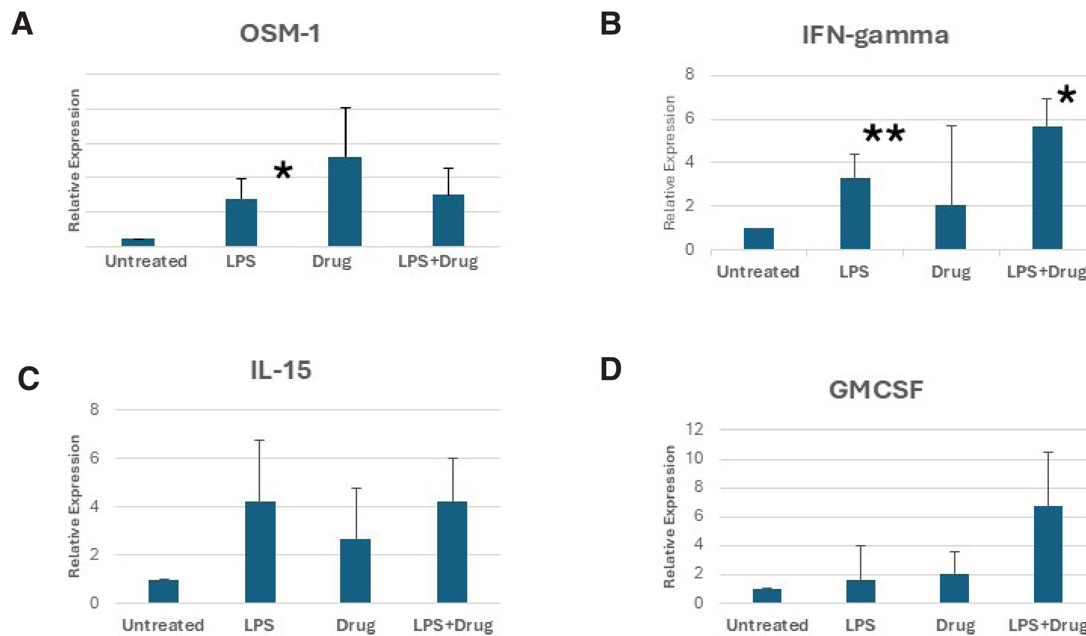

*Fig. 3: Effects of LPS, budesonide, and LPS + budesonide treatment on HepG2 for 24 hrs. The levels of (A) OSM-1, (B) IFN-γ, (C) IL-15, and (D) GMCSF were analyzed by RT-PCR. Results are presented as the mean and ±standard deviation of five independent experiments. * represents statistically significant differences in the level of cytokine expression between treatments compared to the untreated cells. *P<0.01, **P=0.008 vs. control group. The Y axis represents the expression level of the cytokines calculated by the Delta-delta ct method ($2^{-\Delta\Delta Ct}$).*

Conclusion

This study confirms that HepG2 cells in culture secrete OSM-1 and IFN-γ, IL-15, and GMCSF when treated with LPS. However, we were unable to replicate the results of Ma. C. Gutiérrez-Ruiz et al. [24] reported the induction of TNF-α and TGF-beta at the mRNA level and secretion of TGF-beta and IL-1beta cytokines. The results obtained from the analysis of Abcam cytokine arrays suggest that budesonide suppresses the induction of IFN-γ, IL-15, and GM-CSF at the protein level. The data in Figure 3 shows that adding budesonide and LPS together gives a different result at the RNA level. Co-treatment with budesonide and LPS appears to increase the expression of IFN-γ by twofold, which is consistent with previous reports indicating that glucocorticoids can induce certain cytokines depending on the cell type [32]. These contrasting reports suggest that cell type may influence inflammatory cytokine expression in response to budesonide. Budesonide has been reported to have variable effects on cytokine expression depending on cell type and co-stimulatory stimuli [33, 34, 35]. The analysis of cytokine IL-3, IL-1 alpha, and I-309 expression at the RNA level was not conducted. IL-2 showed inconsistent results in PCR.

The suppression of induced cytokines by budesonide remains unclear based on the current data. To more fully elucidate these effects, future research should include a broader range of budesonide concentrations. Furthermore, exploring budesonide's interactions with a variety of inflammatory stimuli capable of inducing cytokine expression could provide crucial insights into its potential therapeutic applications. Given the unclear and inconsistent cytokine response observed in HepG2 cells exposed to LPS and budesonide, it is plausible that this cell line may not constitute the most optimal model for investigating cytokine expression dynamics. A more complex experimental design, such as a co-culture system integrating HepG2 cells with primary immune cells like monocytes or PBMCs, could offer a more physiologically relevant environment to assess budesonide's anti-inflammatory efficacy. By including these refinements in future studies, a more comprehensive understanding of budesonide's therapeutic potential can be achieved. Our study offers novel insights into the intricate interplay between LPS-induced inflammation and the modulatory effects of budesonide within the context of HepG2 cells. Our findings illuminate the necessity for further investigation into the precise molecular mechanisms governing these effects. A deeper understanding of the signaling pathways involved, such as the NF-κB cascade, is imperative for unraveling the complexities of budesonide's therapeutic actions. By understanding these mechanisms, the development of more targeted and efficacious anti-inflammatory strategies could be developed. Moreover, our findings underscore the importance of carefully considering the limitations of cell-based models, such as HepG2 cells, in recapitulating the full spectrum of in vivo inflammatory responses. Future studies incorporating more complex models, such as co-culture systems or animal models, are warranted to bridge the gap between in vitro observations and potential clinical translation.

Ultimately, our research contributes to the growing body of knowledge surrounding the therapeutic potential of budesonide in inflammatory diseases while highlighting the need for continued exploration to fully realize its clinical utility.

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Disclosures. The authors declare no conflicts of interest.

References

[1] F. Balkwill and A. Mantovani, "Inflammation and cancer: back to Virchow?" *The Lancet*, vol. 357, no. 9255, pp. 539–545, Feb. 2001, doi: 10.1016/S0140-6736(00)04046-0.

[2] L. M. Coussens and Z. Werb, "Inflammation and cancer," *Nature*, vol. 420, no. 6917, pp. 860–867, Dec. 2002, doi: 10.1038/nature01322.

[3] S. I. Grivennikov, F. R. Greten, and M. Karin, "Immunity, Inflammation, and Cancer," *Cell*, vol. 140, no. 6, pp. 883–899, Mar. 2010, doi: 10.1016/j.cell.2010.01.025.

[4] F. R. Greten and S. I. Grivennikov, "Inflammation and Cancer: Triggers, Mechanisms, and Consequences," *Immunity*, vol. 51, no. 1, pp. 27–41, Jul. 2019, doi: 10.1016/j.immuni.2019.06.025.

[5] Y. Ma et al., "Anticancer Chemotherapy-Induced Intratumoral Recruitment and Differentiation of Antigen-Presenting Cells," *Immunity*, vol. 38, no. 4, pp. 729–741, Apr. 2013, doi: 10.1016/j.immuni.2013.03.003.

[6] F. Colotta, P. Allavena, A. Sica, C. Garlanda, and A. Mantovani, "Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability," *Carcinogenesis*, vol. 30, no. 7, pp. 1073–1081, Jul. 2009, doi: 10.1093/carcin/bgp127.

[7] H. Zhao et al., "Inflammation and tumor progression: signaling pathways and targeted intervention," *Sig Transduct Target Ther*, vol. 6, no. 1, p. 263, Jul. 2021, doi: 10.1038/s41392-021-00658-5.

[8] S. M. Crusz and F. R. Balkwill, "Inflammation and cancer: advances and new agents," *Nat Rev Clin Oncol*, vol. 12, no. 10, pp. 584–596, Oct. 2015, doi: 10.1038/nrclinonc.2015.105.

[9] S. Zappavigna *et al*., “Anti-Inflammatory Drugs as Anticancer Agents,” *IJMS*, vol. 21, no. 7, p. 2605, Apr. 2020, doi: 10.3390/ijms21072605.

[10] P. M. Rothwell, F. G. R. Fowkes, J. F. Belch, H. Ogawa, C. P. Warlow, and T. W. Meade, “Effect of daily aspirin on long-term risk of death due to cancer: analysis of individual patient data from randomised trials,” *The Lancet*, vol. 377, no. 9759, pp. 31–41, Jan. 2011, doi: 10.1016/S0140-6736(10)62110-1.

[11] E. Eisenberg, C. S. Berkey, D. B. Carr, F. Mosteller, and T. C. Chalmers, “Efficacy and safety of nonsteroidal antiinflammatory drugs for cancer pain: a meta-analysis.,” *JCO*, vol. 12, no. 12, pp. 2756–2765, Dec. 1994, doi: 10.1200/JCO.1994.12.12.2756.

[12] S. D. Reichardt *et al*., “The Role of Glucocorticoids in Inflammatory Diseases,” *Cells*, vol. 10, no. 11, p. 2921, Oct. 2021, doi: 10.3390/cells10112921.

[13] M. Yasir, A. Goyal, and S. Sonthalia, “Corticosteroid Adverse Effects,” StatPearls, Treasure Island (FL): StatPearls Publishing, 2024. [Online]. Available: http://www.ncbi.nlm.nih.gov/books/NBK531462/

[14] S. O’Donnell and C. A. O’Morain, “Therapeutic benefits of budesonide in gastroenterology,” *Therapeutic Advances in Chronic Disease*, vol. 1, no. 4, pp. 177–186, Nov. 2010, doi: 10.1177/2040622310379293.

[15] M. P. Manns, E. Jaeckel, and R. Taubert, “Budesonide in Autoimmune Hepatitis: The Right Drug at the Right Time for the Right Patient,” *Clinical Gastroenterology and Hepatology*, vol. 16, no. 2, pp. 186–189, Feb. 2018, doi: 10.1016/j.cgh.2017.11.003.

[16] S. Hanauer, W. J. Sandborn, A. Persson, and T. Persson, “Budesonide as maintenance treatment in Crohn’s disease: a placebo-controlled trial,” *Aliment Pharmacol Ther*, vol. 21, no. 4, pp. 363–371, Feb. 2005, doi: 10.1111/j.1365-2036.2005.02338.x.

[17] M. N. Ishak, N. Md Shukri, and R. R. Ramli, “The Effectiveness of Budesonide Nasal Irrigation in Patients with Allergic Rhinitis,” *Malays J Med Sci*, vol. 29, no. 1, pp. 34–42, Feb. 2022, doi: 10.21315/mjms2022.29.1.4

[18] G. Veronesi et al., “Long-term effects of inhaled budesonide on screening-detected lung nodules,” *Annals of Oncology*, vol. 26, no. 5, pp. 1025–1030, May 2015, doi: 10.1093/annonc/mdv064.

[19] A. P. Machado et al., “Outcomes of Budesonide as a Treatment Option for Immune Checkpoint Inhibitor-Related Colitis in Patients with Cancer,” *Cancers*, vol. 16, no. 10, p. 1919, May 2024, doi: 10.3390/cancers16101919.

[20] I. N. Crispe, “The Liver as a Lymphoid Organ,” *Annu. Rev. Immunol*., vol. 27, no. 1, pp. 147–163, Apr. 2009, doi: 10.1146/annurev.immunol.021908.132629.

[21] C. O’Farrelly and I. N. Crispe, “Prometheus through the looking glass: reflections on the hepatic immune system,” *Immunology Today*, vol. 20, no. 9, pp. 394–398, Sep. 1999, doi: 10.1016/S0167-5699(99)01518-2.

[22] J. M. Llovet *et al*., “Hepatocellular carcinoma,” *Nat Rev Dis Primers*, vol. 7, no. 1, p. 6, Jan. 2021, doi: 10.1038/s41572-020-00240-3.

[23] M. W. Robinson, C. Harmon, and C. O’Farrelly, “Liver immunology and its role in inflammation and homeostasis,” *Cell Mol Immunol*, vol. 13, no. 3, pp. 267–276, May 2016, doi: 10.1038/cmi.2016.3.

[24] Ma. C. Gutiérrez-Ruiz *et al*., “Cytokines, growth factors, and oxidative stress in HepG2 cells treated with ethanol, acetaldehyde, and LPS,” *Toxicology*, vol. 134, no. 2–3, pp. 197–207, Jun. 1999, doi: 10.1016/S0300-483X(99)00044-X.

[25] J. Chen *et al*., “Inflammation Induced by Lipopolysaccharide and Palmitic Acid Increases Cholesterol Accumulation via Enhancing Myeloid Differentiation Factor 88 Expression in HepG2 Cells,” *Pharmaceuticals*, vol. 15, no. 7, p. 813, Jun. 2022, doi: 10.3390/ph15070813.

[26] G. Panahi, P. Pasalar, M. Zare, R. Rizzuto, and R. Meshkani, “High glucose induces inflammatory responses in HepG2 cells via the oxidative stress-mediated activation of NF-κB, and MAPK pathways in HepG2 cells,” *Archives of Physiology and Biochemistry*, vol. 124, no. 5, pp. 468–474, Oct. 2018, doi: 10.1080/13813455.2018.1427764.

[27] “Dojindo Laboratories Store”. [Online]. Available: https://www.dojindo.eu.com/store/AccountOnHold.aspx

[28] X. Rao, D. Lai, and X. Huang, “A New Method for Quantitative Real-Time Polymerase Chain Reaction Data Analysis,” *Journal of Computational Biology*, vol. 20, no. 9, pp. 703–711, Sep. 2013, doi: 10.1089/cmb.2012.0279.

[29] X. Liu *et al*., “LPS induced proinflammatory cytokine expression in human airway epithelial cells and macrophages via NF κB, STAT3 or AP 1 activation,” *Mol Med Report*, Feb. 2018, doi: 10.3892/mmr.2018.8542.

[30] D. Meir and B. Hanna, “Budesonide Modulates the Immune Cross-Talk between Mononuclear and Colon Cancer Cells,” *Int J Immunol Immunother*, vol. 5, no. 1, Dec. 2018, doi: 10.23937/2378-3672/1410029.

[31] F. Paolieri *et al*., “Inhibition of adhesion molecules by budesonide on a human epithelial cell line (lung carcinoma),” *Allergy*, vol. 52, no. 9, pp. 935–943, Sep. 1997, doi: 10.1111/j.1398-9995.1997.tb01254.x.

[32] J. M. Ehrchen, J. Roth, and K. Barczyk-Kahlert, “More Than Suppression: Glucocorticoid Action on Monocytes and Macrophages,” *Front. Immunol*., vol. 10, p. 2028, Aug. 2019, doi: 10.3389/fimmu.2019.02028.

[33] S. Rennard *et al*., “Anti-inflammatory effects of budesonide in human lung fibroblasts are independent of histone deacetylase 2,” *JIR*, p. 109, Aug. 2013, doi: 10.2147/JIR.S43736.

[34] R. Leigh *et al.*, “An inhaled dose of budesonide induces genes involved in transcription and signaling in the human airways: enhancement of anti- and proinflammatory effector genes,” *Pharmacology Res & Perspec*, vol. 4, no. 4, p. e00243, Aug. 2016, doi: 10.1002/prp2.243.

[35] I. Von Scheele, K. Larsson, and L. Palmberg, “Budesonide enhances Toll-like receptor 2 expression in activated bronchial epithelial cells,” *Inhalation Toxicology*, vol. 22, no. 6, pp. 493–499, May 2010, doi: 10.3109/08958370903521216.