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Niclosamide Modulates the Cellular and Humoral Immune Response in Balb/c Mice

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ABSTRACT

Background: Niclosamide, a STAT3 inhibitor, is widely under investigation due to its anti-cancer properties. STAT3 also exhibits an exciting role in the immune responses.

Objective: This study aimed to evaluate the impact of niclosamide on immune response of mice.

Methods: Niclosamide was administered to balb/c mice. To evaluate cell-mediated immune response, a contact-hypersensitivity (CHS) test, cyclophosphamide-induced neutropenic assay, and carbon clearance test were performed, whereas a humoral immune response is evaluated by hemagglutination assay (HA) and mice lethality test. The concentration of TGF-β1 was determined by enzyme-linked immunosorbent assay (ELISA) on murine peritoneal macrophages.

Results: In the CHS test, niclosamide caused a decrease in skin thickness, significantly exhibiting a decrease in inflammation. A highly significant decrease in overall leukocyte count (lymphocytes and neutrophils) was observed before and after cyclophosphamide injection as compared with the control group. However, only a highly significant decrease in the neutrophil percentage was observed. Niclosamide has decreased the phagocytic process immensely compared with the control. In the HA titer, niclosamide was found to reduce the antibodies' titer compared with the negative control group. In the mice lethality test, the treatment groups have shown an increase in the percentage of mortality. TGF-β1 elevated in peritoneal macrophages when treated with niclosamide, in a dose-dependent manner.

Conclusion: Niclosamide exerts potent immunomodulatory effects by significantly suppressing cell-mediated and humoral immune responses and increasing the levels of TGF-β1 in mice. Niclosamide might be added as an adjuvant to immunosuppressive drugs for the treatment of autoimmune diseases.

Keywords: Cellular Immunity, Humoral Immunity, Immunomodulation, Niclosamide, TGF-β1

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INTRODUCTION

Immunomodulation is an intervention aimed to modify the host's immune response to achieve the desired therapeutic effect. Modulation of immune response is of immense importance, especially following an infection or vaccine administration. [1]. Therefore, administering an immune-stimulant agent may help in an elevated immune response in vaccinated individuals. On the contrary, an immunosuppressive agent may lead to further suppression of immune response, giving rise to parasitic infections [2]. Following infection, the innate and adaptive immune system come into play. Cell-mediated immune response and humoral immune response are the two major components of the adaptive immune system. The immune response mediated by the cellular immune system mainly comprises macrophages, helper T-cells (T_H), natural killer (NK) cells, and CD8+ T cells (also called cytotoxic T lymphocytes or CTLs), whereas the humoral immune response involves the production of antibodies. Transforming growth factor-beta 1 (TGF-β1) plays a vital role in the regulation of the immune system and displays a dual effect on the immune system [3]. The role of TGF-β1 on the immune response is largely based upon the other secreted factors. TGF-β1 may either suppress adaptive immunity by suppressing T_H1 cells, T_H2 cells, and CD8+ T-cells or enhance adaptive immunity by induction of CD4+ T-cells, T_H9 cells, and T_H17 cells [4].

Niclosamide is a Food and Drug Administration (FDA) approved anthelmintic drug. For decades, it has been in use for its anticestodals activity in humans and veterinary, equally [5]. Once the drug of choice in the treatment of *Taenia solium* (pork tapeworm) and *Taenia saginata* (beef tapeworm) infections, it is still widely used in veterinary and agriculture as an anthelmintic and a pesticide, respectively [6]. The drug performs its anthelmintic action by inhibiting anaerobic Adenosine triphosphate (ATP) production and mitochondrial oxidative phosphorylation in these parasites [7]. It has a very well-documented safety profile, and is still a part of the essential medicine list of the World Health Organization (WHO) [8]. Recent studies suggest that niclosamide has a wide variety of anticancer, antioxidant, and antiviral activities [9]. Niclosamide exhibits multifunctional properties and its ability to inhibit the selective signal transducer and activator of transcription 3 (STAT3) has made it an exciting candidate in the modulation of cancers and immune responses [10]. It is believed that the elimination of STAT3 has a role in the prevention of autoimmune diseases [11]. Other studies have also suggested that niclosamide has modulated the immune responses by affecting cytokines and inflammatory activity of dendritic cells (DCs) [12], it has also impressively modulated the activity of the immune system by affecting the levels of the interleukins [13]. In the disease model, niclosamide was found to exhibit beneficial immunomodulatory activity [14].

Since drug repurposing is a preferable method to evaluate the new pharmacological use of an old drug [15], hence this study was designed to investigate the effects of niclosamide on the immune response, as some recent studies reported that niclosamide has the potential to affect the key players of the immune system.

MATERIALS AND METHODS

Reagents

Niclosamide (purity>95%) was acquired from Shaigan Pharmaceuticals Pvt. Ltd., Rawalpindi, Pakistan. Dimethyl sulfoxide (DMSO), phosphate buffer saline (PBS), water for injection, 2,4-Dinitrochlorobenzene (DNCB), Carboxymethyl cellulose (CMC), Indian dispersion ink were obtained from the Department of Pharmacology and Toxicology, the University of Veterinary and Animal Sciences (UVAS). The hemorrhagic septicemia vaccine was acquired from the

Veterinary Research Institute, Lahore. Powder for injection of cyclophosphamide was purchased from Pharmedic Pvt. Ltd. Enzyme-linked immunosorbent assay (ELISA) kit for TGF-β1 was purchased from Cloud-Clone Corp. (SEA124Mu), having a sensitivity of 6.4 pg/ml and a detection range of 15.6 pg/ml to 1000 pg/ml.

Experimental Animal and Protocol

Inbred male Balb/c mice, aged 5-8 weeks, and weight 28.45±1.78 grams were used. These mice were purchased from the Department of Theriogenology, UVAS. The mice were kept in the animal house of UVAS and all the institutional protocols were kept under consideration throughout the study. Prior ethical approval was taken from the Office of Research Innovation and Commercialization (ORIC), UVAS (Letter No. DR/1951). Mice were given a standard pellet diet daily and clean drinking water *ad libitum*.

For cell-mediated immunity, contact hypersensitivity (CHS), carbon clearance test, and cyclophosphamide-induced neutropenia assay were employed, whereas humoral immunity was evaluated by mice lethality test and indirect hemagglutination assay (IHA). Three different doses of niclosamide (10 mg/kg/day, 20 mg/kg/day, and 4 0mg/kg/day) were administered daily as a single dose via the intraperitoneal route. It was dissolved in 1% DMSO and diluted with PBS solution immediately before injecting the mice. Cyclophosphamide was administered as a reference drug throughout the study to compare the immunosuppressive effect of niclosamide. Cyclophosphamide is a chemotherapeutic agent possessing a marked ability to induce immune suppression. Cyclophosphamide causes the alkylation of DNA of bone marrow cells, thus leading to immunosuppression [16]. Hence, it is an ideal candidate to serve as a control in immunomodulatory studies of a new drug. The TGF-β activity was analyzed by performing ELISA on peritoneal macrophages isolated from mice. A dose of 0.3125µM, 0.625µM, and 1.25µM was used by dissolving niclosamide in 0.1% DMSO.

Contact Hypersensitivity (CHS) Test

The mice were divided into five groups, each having five mice (n=25). On the 1st day of the trial, 150mg/kg cyclophosphamide was administered subcutaneously to the positive control group. Cyclophosphamide served as a reference drug since cyclophosphamide induces profound immunosuppression in the mice. The negative control was administered 1% DMSO solution and the rest of the three groups were administered different doses of niclosamide intraperitoneally. On the second day of the trial, hair was shaved from the left side of the mice's abdomen, and a sensitizing dose of 0.2 ml 2% DNCB was applied in all the groups topically. 2 grams DNCB was dissolved in a small quantity of acetone and then the volume was made up to 100ml with acetone. On the 8th day, hair was shaved from the right side, and measurements were taken using a digital Vernier caliper. This measurement was labeled as baseline skin thickness. Later, the mice were challenged with 0.2 ml 1% DNCB. Skin thickness measurements were taken after 24, 48, and 72 hrs. [17]. The changes in skin thickness were measured by subtracting baseline skin thickness from skin thickness after 24 hrs [18]. Results were presented as a percentage suppression of contact hypersensitivity using the formula below:

$$Percentage\ suppression = \left(1 - \frac{\Delta T_E}{\Delta T_s}\right) \times 100\%$$

Where, ΔT_E represents the mean change in skin thickness over 24 hours period in the experimental group whereas, ΔT_s represents the skin thickness in the negative control group.

Cyclophosphamide-Induced Neutropenic Assay

This assay was performed on four groups of mice (n=20). The control group was administered a 1% DMSO solution, whereas the treatment groups were injected with the

three doses of niclosamide till the 9th day of the trial. On the 10th day, the blood was collected via cardiac puncture and stored in vacutainers containing EDTA. Differential leukocyte count (DLC) and total leukocyte count (TLC) were calculated in this blood sample, and the mice were injected subcutaneously with a neutropenic dose of cyclophosphamide (200 mg/kg) on the same day. A complete blood count (CBC) was performed by an automated CBC analyzer at Pet Center, UVAS. Moreover, a thin smear of the blood sample was prepared on a glass slide, neutrophils were stained with Geimsa stain and the number of neutrophils was counted using a Neubauer chamber. On the 13th day, the blood was again collected, and the percentage of the depletion in TLC and DLC calculations were made [19, 20].

The percentage of the depletion of the DLC and TLC was calculated using the following formulae:

$$Percentage\ reduction = \frac{No.of\ cells\ before\ cyclophosphamide - No.of\ cells\ after\ cyclophosphamide}{No.of\ cells\ before\ cyclophosphamide} \times 100$$

Carbon Clearance Test

To assess the activity of phagocytes, carbon clearance test was carried out. Niclosamide was injected intraperitoneally into the three treatment groups. The control group was given 0.5% CMC per oral (10 ml/kg). On the 7th day of the trial, the mice were weighed again, and all the mice were injected intravenously with Indian ink (0.1 ml ink for a 30-gram mouse). The blood was collected from the mice at the time of injection of Indian ink (*t=0 mins*). Blood was again recollected from each mouse after 15 minutes (t=15 mins). The mice were sacrificed by cervical dislocation and their spleen, and liver were isolated and weighed. For the lysis of erythrocytes, 2 ml of 0.1% of sodium carbonate (Na_2CO_3) solution was added to 50 μl of mice blood samples. Spectrophotometric analysis of this solution was performed for a percentage absorbance of samples at 650 nm [21-23].

The following formulae were used to calculate the Carbon clearance rate:

$$Rate\ of\ carbon\ clearance\ (K) = \frac{log\ OD_0 - log\ OD_{15}}{T_2 - T_1}$$

$$Phagocytic\ index\ (\alpha) = \frac{body\ weight\ of\ mice\ \times \sqrt[3]{K}}{Weight\ of\ spleen + liver}$$

Indirect Hemagglutination Assay (IHA)

The negative control group was administered 0.2 ml of 1% DMSO solution. Cyclophosphamide (150 mg/kg/7 days) was injected into the positive control group. The treatment groups were administered three different doses of niclosamide, five days a week for 28 days, respectively [13, 24]. Whereas, group 4 was kept as the negative control and was injected with 0.2 ml of the vehicle (1% DMSO solution). On the 14th day, 0.1 ml (0.5×10^8/ml) of the sheep red blood cells (SRBCs) collected from a healthy sheep were injected intraperitoneally into the mice's abdominal cavity. A booster dose of 0.1 ml of SRBCs was injected on the 21st day of the test. On both occasions, the SRBCs were collected from the same sheep housed at the animal shed of the University of Veterinary and Animal Sciences. After seven days of the booster dose, the blood was collected in tubes via retro-orbital puncture. These tubes were then centrifuged, and the resulting supernatant was separated to obtain purified serum. HA titer was performed, and antibody titer values were calculated against SRBCs [20, 25].

Mice Lethality Test

Each group held five mice (n=25), the positive control group was injected with cyclophosphamide subcutaneously, whereas the negative control group or vehicle group was treated with 1% DMSO solution. The rest of the groups were administered niclosamide for 21 days in three different doses. On the 7th and 17th days, all the groups were vaccinated with the hemorrhagic septicemia vaccine. A challenging dose of *Pasteurella multocida* culture was administered to all the mice according to their weight through the subcutaneous route on the 21st day of assay. Mice mortality was noted for the next 72 hrs.

and the percentage mortality was calculated by the formula given below [17, 26].

$$Mortality\ percentage = \frac{Number\ of\ mice\ dead}{Total\ number\ of\ mice} \times 100$$

Isolation of Peritoneal Macrophages

Inbred male Balb/c mice (n=35) were purchased from the Department of Theriogenology, UVAS. After adaptation to the laboratory environment, macrophages from mice were isolated by peritoneal lavage with ice-cold PBS. The cell suspension was centrifuged at 1500 RPM for 8 minutes, and the supernatant was discarded. A portion of the cells was suspended in the RPMI media and these cells were stained with trypan blue stain and counted by using a hemocytometer. Cells were washed and suspended in RPMI-1640 medium containing 10% fetal bovine serum (FBS). This medium was cultured in 6-well plates and then incubated at 37°C for 3 hrs. in a 5% CO_2 incubator [27, 28]. The adherent cells were used in the study.

Determination of TGF-β1

The incubated peritoneal macrophages were adjusted at 1×10^6 cells/well. Thet treatment groups were treated with 0.3125 µM, 0.625 µM, and 1.25 µM of niclosamide, whereas the vehicle group was treated with 0.1% DMSO solution. Plates were again incubated at 37°C in a 5% CO_2 incubator for 24 hrs. The supernatant was removed and centrifuged at 1000 RPM for 10 minutes. The resulting supernatant was isolated and stored at -20°C. ELISA was performed, and TGF-β1 concentration was measured as stated in the manufacturer's instruction manual [27, 29].

Statistical Analysis

Each experiment was repeated thrice with similar results (N=3). Statistical Package for the Social Sciences (SPSS Inc. v21) was employed to analyze the collected data. The mean and percentages exhibited the descriptive results. The Shapiro-Wilk test was applied to assess the normality of the data. For inferential statistics, one-way ANOVA was used mainly, followed by Bonferroni posthoc analysis. Contact hypersensitivity results were analyzed by Student's t-test. However, for qualitative results, the Chi-square test was applied. A P-value of less than 0.05 was considered significant.

RESULTS

Contact Hypersensitivity (CHS) Test

The negative control group exhibited a constant increase in mean skin thickness at 24, 48, and 72 hrs. In the positive control group, an increase in skin thickness was observed until 24 hrs. after the administration of cyclophosphamide. Percent suppression of contact hypersensitivity was evaluated and was found to have a dose-dependent response to niclosamide. At the dose of 10 mg/kg and 20 mg/kg, the percentage suppression was non-significant and was found to be 25% and 38.9%, respectively. Conversely, 40 mg/kg of niclosamide, the reference drug, and cyclophosphamide showed a significant difference ($P<0.05$) in suppressing the hypersensitivity reaction. Furthermore, niclosamide (40 mg/kg) and cyclophosphamide were found to cause percentage suppression of 55.6% and 61.1%, respectively. (Figure 1)

Cyclophosphamide-induced Neutropenia Assay

A dose-dependent decrease in the percentage of TLC and neutrophil count was observed (Table 1). A percentage decrease in the control group when compared with treatment groups suggested a significant percentage decrease of 77.9% and 79.5% at the doses of 20, and 40 mg/kg of niclosamide, respectively (*$P<0.05$). In general, a decrease in total leukocytes, lymphocytes, and neutrophils was observed after the administration of cyclophosphamide on the 10th day of the trial.

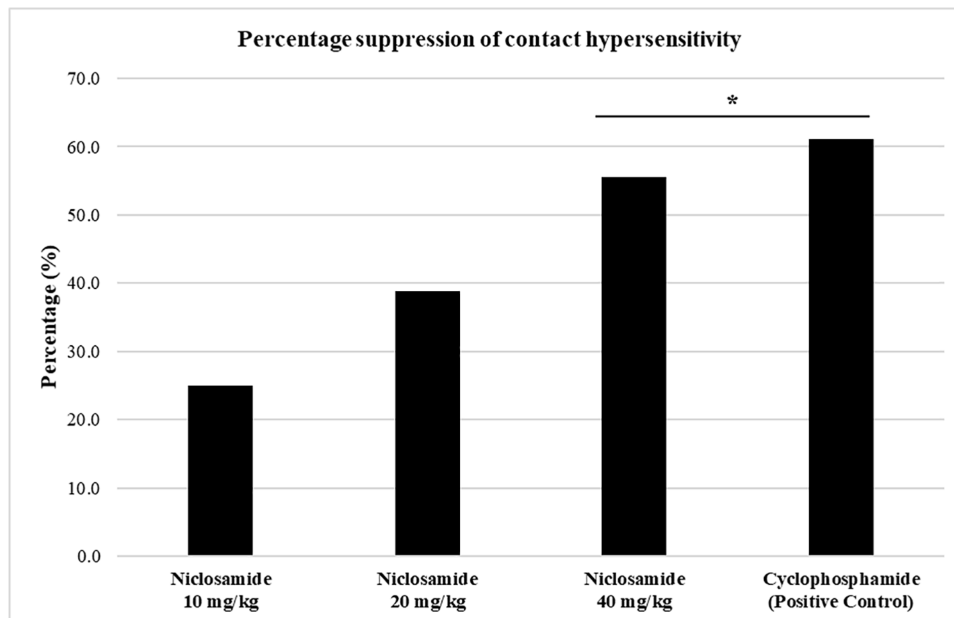

Figure 1. Percentage suppression of contact hypersensitivity. *P<0.05 indicates a significant difference

Table 1. Mean cells count before and after administration of cyclophosphamide, and percentage reduction in TLC and neutrophils

	Total Leukocyte count (TLC)			Neutrophil count		
	Before (cells/ µL)±SEM	After (cells/ µL)±SEM	Percentage Reduction	Before (cells/ µL)±SEM	After (cells/ µL)±SEM	Percentage Reduction
Control Group (n=5)	6858±173.0	3580±242.0	47.8%	1577±139.8	783±30.4	50.3%
Niclosamide 10mg/kg (n=5)	5320±297.2	2340±140.0	56.0%	696±25.1	226±26.0	67.5%
Niclosamide 20mg/kg (n=5)	3868±212.1	1660±92.7	57.1%	456±25.3	101±20.2	77.9%*
Niclosamide 40mg/kg (n=5)	2930±339.9	1020±166.0	65.2%	269±67.0	55±11.3	79.5%*

*P<0.05; n=number of mice in each group; SEM=Standard Error of Mean

Carbon Clearance Test

The average values of the rate of a carbon clearance and phagocytic index among different test groups were calculated. The mean phagocytic index in the group given CMC was found to be 5.539 (Table 2). The treatment groups have shown a dose-dependent decrease in phagocytic activity (P<0.05). Here, niclosamide at the dose of 10mg/kg, 20mg/kg, and 40mg/kg had been shown to decrease the phagocytic index to 4.068, 3.264, and 2.168, respectively. Pairwise comparison showed a significant decrease in the phagocytic index at a dose of 40 mg/kg of niclosamide only (*P<0.05).

Indirect Hemagglutination Assay

Niclosamide has shown a dose-dependent decrease in the log HA titer of the treatment groups. The mean log HA titer in the positive control group was 2.14, whereas the negative control group had a value of 7.58 (Table 3). Niclosamide at the higher doses exhibited a highly significant decrease in the log HA titer (**P<0.001).

Mice Lethality Test

An overall mortality rate within 24, 48,

Table 2. Average rate of carbon clearance and average phagocytic index in treated mice

Group	Average Rate of carbon clearance (k±SEM)	Average phagocytic index (α±SEM)
Control Group (n=5)	0.019±0.001	5.539±0.504
Niclosamide 10 mg/kg (n=5)	0.012±0.006	4.068±0.979
Niclosamide 20 mg/kg (n=5)	0.004±0.001*	3.264±0.580
Niclosamide 40 mg/kg (n=5)	0.001±0.001*	2.168±0.265*

*P<0.05; n=number of mice in each group; SEM=Standard Error of Mean; k=Rate of carbon clearance; α=Phagocytic index

Table 3. Logarithmic values of mean hemagglutination (HA) titer of niclosamide treated groups and positive control group in comparison with the negative control group.

Groups	$\log_2$ values of mean HA titer±SEM
Positive control (Cyclophosphamide) (n=5)	2.14±0.49***
Negative control (1% DMSO+PBS) (n=5)	7.58±0.40
Niclosamide 10 mg/kg/day (n=5)	5.85±0.60
Niclosamide 20 mg/kg/day (n=5)	3.07±0.49***
Niclosamide 40 mg/kg/day (n=5)	2.26±0.45***

***P<0.001; n=number of mice in each group; SEM=Standard Error of Mean

and 72 hrs. was calculated and recorded. In the negative and positive control groups, the percentage mortality was 20% and 100%, respectively. However, the groups injected with niclosamide at the dose of 10 mg/kg, 20 mg/kg and 40 mg/kg had percentage mortality of 40%, 60%, and 100%, respectively. Hence dose-dependent percentage mortality was observed in the treatment groups. The Chi-square test was applied, followed by Cramer's V value to assess the association of mortality with various groups. A significant association was found between the mortality and treatment groups (*P<0.05). A Cramer's V value suggests that there is a strong relationship between the treatment groups and mortality ratio (P<0.05).

Determination of TGF-β1

The results (Figure 2) expressed a dose-dependent increase in the concentration of TGF-β1 in niclosamide-treated groups in comparison with the vehicle group. The concentration of TGF-β1 in the vehicle group was found to be 17.42±0.07 pg/ml, whereas niclosamide at a dose of 0.3125 µM, 0.625 µM, and 1.25 µM increased the concentration of TGF-β1 to 21.70±0.16 pg/ml, 29.06±0.09 pg/ml, and 57.39±0.22 pg/ml, respectively (*P<0.05).

DISCUSSION

Delayed-type hypersensitivity evaluates the activity of cell-mediated immune response. Delayed-type hypersensitivity reactions are also classified as Type IV hypersensitivity reactions and are usually triggered by T_H1 cells [30]. DNCB is a hapten and is used as an irritant as it forms a dinitrophenyl complex and serves as an antigen leading to the induction of a hypersensitivity reaction. Niclosamide (40 mg/kg) decreased the skin thickness significantly, exhibiting a decrease in inflammation. This decrease shows that niclosamide has prevented the inflammatory response that may have otherwise resulted due

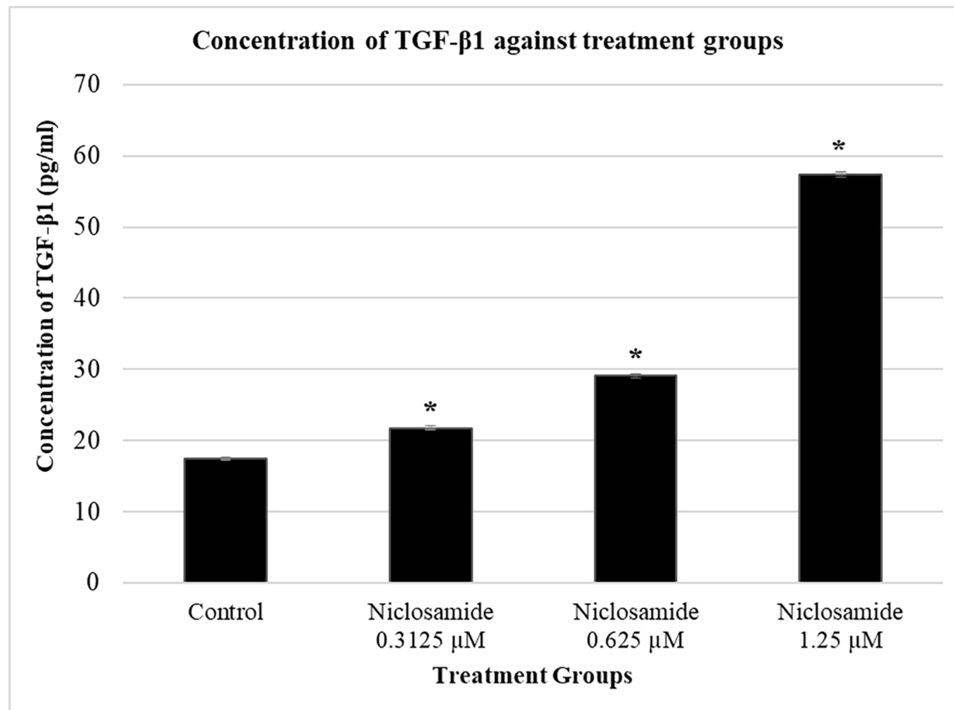

Figure 2. Stimulatory effects of niclosamide on the concentration of TGF-β1 in comparison with the control group. Data are mean±SEM. *P<0.05 indicates significant differences from the control group

to the triggered cellular immune response. The finding of our study is similar to another study where niclosamide has attenuated the response of contact hypersensitivity reaction induced by 2,4-dinitro 1-fluorobenzene (DNFB) [12]. Niclosamide may exert its anti-inflammatory effect by reducing the secretion of pro-inflammatory cytokines (IL-1, TNF-α, and IFN-γ) [31]. The drug may also exert this effect by inhibiting IL-12. IL-12 is a cytokine responsible for the conversion of naïve CD4 cells into T_H1 cells, which in turn release cytokines (IL-1, TNF-α, and INF-γ) and helps with the cell-mediated immune response [32]. Our results represent niclosamide which causes the suppression of a cell-mediated immune response; hence, it may be a useful treatment option in inflammatory disorders or conditions such as contact dermatitis.

Cyclophosphamide is one of the most profoundly used drugs to induce neutropenia. In this study, a low dose of cyclophosphamide was used to induce neutropenia. Cyclophosphamide exhibits its leukopenic property by interfering with DNA replication [33]. Cyclophosphamide can reduce all the proliferative cells responsible for the immune response [34]. The proliferation of neutrophils from their progenitors is regulated by Granulocyte colony-stimulating factor (G-CSF) [35]. The G-CSF binding to its receptors activates STAT3. This pathway is not solely responsible for the differentiation of myeloid cells into neutrophils. However, STAT3 activation is the main reason for the differentiation of neutrophils [36]. In this view, the cyclophosphamide-induced neutropenic assay was performed. A highly significant decrease in overall leukocyte count (lymphocytes and neutrophils) was observed before and after cyclophosphamide injection in comparison with the negative control group. Neutrophils are the cells of the innate immune system that play their roles by phagocytosis [37]. Hence, the reduction in neutrophils suggests a decrease in cell-mediated immune response after the administration of niclosamide. The G-CSF, along with STAT3, is responsible for the mobilization of neutrophils in circulation. The inhibition of STAT3 by niclosamide may have resulted in decreased neutrophil

migration in systemic circulation; therefore, the percentage decrease was evident in our results [38].

The Reticuloendothelial system (RES) has a significant role in the clearance of any foreign particle from blood, including inert substances and dyes [23]. Macrophages are a vital component of the innate and adaptive immune system and perform their action by phagocytosis [39]. The activity of macrophages is enhanced in response to the release of IL-12 and IFN-γ from splenocytes, which in turn activates the release of TNF-α [40]. Indian ink, when injected into the blood, is a source of colloidal carbon particles which upon their contact with macrophages are broken down [22], leading to the conclusion that an increased rate of carbon clearance is correlated to enhanced phagocytic activity [41]. In our study, the effect of niclosamide was evaluated by using the carbon clearance test, and it was found that the drug resulted in a decreased phagocytic index value suggesting suppressed phagocytic activity. A similar study also exhibited the same response as our study, wherein the niclosamide reduced the activity of dendritic cells [12]. It may have performed this action by affecting the expression of major histocompatibility complex (MHC) and reducing the ability of cells to take up the antigen.

A humoral immune response is acquired by the generation of antibodies against the specific antigen. An indirect hemagglutination assay is among the most specific and sensitive serological tests, used to detect the presence of antibodies in the serum sample. B-cells activation on an encounter with an antigen leads to the maturation of themselves into plasma cells. MHC-II regulates the activation of B-cells which in turn acts as antigen-presenting cells and presents the antigen [42]. However, the B-cells get their activating signals from T_H1 cells and $CD4^+$ T-helper 2 cells. In response to a protein antigen, three different types of immunoglobulins (IgA, IgG, IgE) are produced from plasma cells while lipids and polysaccharides lead to the formation of IgM mainly [43]. In our study, the SRBCs served as an antigen, and when injected into mice resulted in the production of antibodies against SRBCs in the blood. IgM is the primary antibody that arises in the blood in response to the injection of SRBCs [19]. Niclosamide was found to reduce the number of antibodies compared with the negative control group. The reduction in the humoral response was dose-dependent, i.e., niclosamide demonstrated a profound decrease in logarithmic HA titer when the dose increased.

Similar results were observed in the study of mice model where, niclosamide reduced the number of T-cells and all the population of B-cells hence, decreasing the number of autoantibodies in mice [13]. This reduction might be because $CD4^+$ T-cells cause the activation of B-cells via STAT3 signals activated by IL-21 and IL-27 [44]. Niclosamide is a potent inhibitor of STAT3 [45], and by inhibiting the phosphorylation of the STAT3 pathway [46], may have inhibited the activation of B-cells and antibodies. Another study demonstrated similar results where niclosamide had reduced the production of memory T-cells ($CD4^+$ and $CD8^+$) and B-cells activation [14]. In a mouse model of systemic sclerosis, niclosamide was found to alleviate the symptoms of the disease [13]. It is suggested that B-cells play a vital role in the progression of the disease. Therefore, the humoral immune response is mainly affected by niclosamide by interacting with B-cells [47].

The mice lethality test is a widely used serological test. In our study, a culture of *Pasteurella multocida* was injected into mice to assess the immunological response of the *Pasteurella multocida* toxin (PMT) in the mice treated with niclosamide and hemorrhagic septicemia (HS) vaccine. *P. multocida* infections are zoonotic; however, in animals, it may lead to hemorrhagic septicemia [48]. The toxin affects the respiratory system and suppresses it, causing animal mortality. The lipopolysaccharide (LPS) on the surface of *P. multocida* on an encounter with host

immune cells (macrophages, dendritic cells, or neutrophils) may bind to the immune cells via a toll-like receptor (TLR-4). This binding generates a signaling cascade leading to the activation of NFκB.

On the other hand, the PMT acts intracellularly and deamidates G-protein, also leading to the activation of transcription factors, i.e., STAT and NFκB [49]. Both of these inducers further release pro-inflammatory cytokines, including IL-1β, IL-6, IL-12, and TNF-α. An organism with a prior administration of the HS vaccine prevents the pathogenicity of bacteria and prevents mortality. However, the groups administered any immunosuppressive drug may result in the mortality of mice. A decrease in the levels of IgG and IgM followed by the administration of *P. multocida* also results in the mortality of the subject [50]. In the trial, the treatment groups have shown a dose-dependent increase in percentage mortality. As discussed above, niclosamide may have exhibited this response since it is an inhibitor of pro-inflammatory cytokines [31, 51]. Since niclosamide is a STAT and NFκB inhibitor, it may have suppressed the humoral immune response [12].

Activated TGF-β1 mainly leads to the suppression in the adaptive and innate immune response [52]. Our results expressed a dose-dependent increase in the concentration of TGF-β1 in response to niclosamide on murine peritoneal macrophages [53]. A similar study evaluating the role of STAT3 on the TGF-β family, suggests an inhibitory expression of TGF-β due to the downregulation of others against decapentaplegic homolog 7 (smad7) induced by STAT3 activation [54, 55]. Since niclosamide is a STAT3 inhibitor, it may have elevated the expression of TGF-β1 by inhibiting STAT3/Smad7/TGF-β signaling. Another study has proposed a similar outcome of Smad7 in the regulation of TGF-β [56].

On the contrary, human trials of Crohns's disease suggest that STAT3 activation may lead to the increased production of TGF- β1 [57]. The role of STAT3 in the regulation of TGF-β1 is still unknown. Our study is valuable as it has depicted an increase in the concentration of TGF-β1 in response to niclosamide, a STAT3 inhibitor. This outcome provides an exciting insight into the further assessment of the role of niclosamide in the immune system and immune-mediated diseases.

CONCLUSION

In summary, niclosamide possesses potent immunomodulating properties by significantly suppressing cell-mediated and humoral immune responses in mice. It also elevated the level of TGF-β1 released by peritoneal macrophages. Niclosamide may be considered as an adjuvant with other immunosuppressive drugs for the treatment of autoimmune diseases and the prevention of graft rejections.

AUTHOR CONTRIBUTION STATEMENT

BM and AQ conceived and designed the research. BM and MA conducted the experiments. AA contributed new reagents or analytical tools. BM and AS analyzed the data. BM wrote the manuscript. MA did the review and approved the final manuscript. All authors read and approved the manuscript and all data were generated in-house and no paper mill was used.

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COMPLIANCE WITH ETHICAL STANDARDS

All the ethical standards were kept in mind throughout the study.

Conflict of Interest: None declared.

REFERENCES

1. Sajid MS, Iqbal Z, Muhammad G, Sandhu MA, Khan MN, Saqib M et al. Effect of ivermectin on the cellular and humoral immune responses of rabbits. Life Sci. 2007; 80: 1966-1970. http://www.ncbi.nlm.nih.gov/pubmed/17379254.
2. Mojžišová J, Hromada R, Paulík Š, Ondrašovič M, Bajová V. Immune response and immunomodulatory effect of levamisole in immunosuppressed dogs vaccinated against parvovirosis. Bull Vet Inst Pulawy. 2004; 48: 93-97.
3. Glick AB, Perez-Lorenzo R, Mohammed J. Context-dependent regulation of cutaneous immunological responses by tgfβ1 and its role in skin carcinogenesis. Carcinogenesis. 2007; 29: 9-14.
4. Travis MA, Sheppard D. Tgf-beta activation and function in immunity. Annu Rev Immunol. 2014; 32. 51-82. http://www.ncbi.nlm.nih.gov/pubmed/24313777.
5. Swan G. The pharmacology of halogenated salicylanilides and their anthelmintic use in animals. J S Afr Vet Assoc. 1999; 70: 61-70.
6. Vermund SH, MacLeod S, Goldstein RG. Taeniasis unresponsive to a single dose of niclosamide: Case report of persistent infection with taenia saginata and a review of therapy. Rev Infect Dis. 1986; 8: 423-426. http://www.ncbi.nlm.nih.gov/pubmed/3726395.
7. Pampori NA, Singh G, Srivastava VM. Energy metabolism in cotugnia digonopora and the effect of anthelmintics. Mol Biochem Parasitol. 1984; 11: 205-213. http://www.ncbi.nlm.nih.gov/pubmed/6749180.
8. Organization WH. Who model list of essential medicines, 18th list (april 2013)(final amendments-october 2013). Geneva; 2013.
9. Liao Z, Nan G, Yan Z, Zeng L, Deng Y, Ye J et al. The anthelmintic drug niclosamide inhibits the proliferative activity of human osteosarcoma cells by targeting multiple signal pathways. Curr Cancer Drug Targets. 2015; 15: 726-738. http://www.ncbi.nlm.nih.gov/pubmed/26118906.
10. Chen W, Mook RA, Jr., Premont RT, Wang J. Niclosamide: Beyond an antihelminthic drug. Cell Signal. 2018; 41: 89-96. http://www.ncbi.nlm.nih.gov/pubmed/28389414.
11. Liu X, Lee YS, Yu CR, Egwuagu CE. Loss of stat3 in cd4+ t cells prevents development of experimental autoimmune diseases. J Immunol. 2008; 180: 6070-6076. http://www.ncbi.nlm.nih.gov/pubmed/18424728.
12. Wu CS, Li YR, Chen JJ, Chen YC, Chu CL, Pan IH et al. Antihelminthic niclosamide modulates dendritic cells activation and function. Cell Immunol. 2014; 288: 15-23. http://www.ncbi.nlm.nih.gov/pubmed/24561310.
13. Morin F, Kavian N, Nicco C, Cerles O, Chéreau C, Batteux F. Niclosamide prevents systemic sclerosis in a reactive oxygen species–induced mouse model. J Immunol. 2016. 1502482.
14. Morin F, Kavian N, Nicco C, Cerles O, Chereau C, Batteux F. Improvement of sclerodermatous graft-versus-host disease in mice by niclosamide. J Invest Dermatol. 2016; 136: 2158-2167. http://www.ncbi.nlm.nih.gov/pubmed/27424318.
15. Oprea TI, Mestres J. Drug repurposing: Far beyond new targets for old drugs. AAPS J. 2012; 14: 759-763. http://www.ncbi.nlm.nih.gov/pubmed/22826034.
16. Shabbir A, Arshad HM, Shahzad M, Shamsi S, Ashraf MI. Immunomodulatory activity of mefenamic acid in mice models of cell-mediated and humoral immunity. Indian J Pharmacol. 2016; 48: 172-178. http://www.ncbi.nlm.nih.gov/pubmed/27127320.
17. Khurram S, Javeed A, Ashraf M, Nazir J, Ghafoor A, Mustafa G. Effect of colchicine on humoral immunity and immune organs in mice. J Anim Plant Sci. 2017; 27 (4).
18. Gaspari AA, Katz SI, Martin SF. Contact hypersensitivity. Curr Protoc Immunol. 2016; 113: 421-427. http://www.ncbi.nlm.nih.gov/pubmed/27038464.
19. Thomas L, Asad M, Hrishikeshavan HJ, Chandrakala GK. Effect of centchroman on cellular and humoral immunity. Indian J Physiol Pharmacol. 2007; 51: 387-394. http://www.ncbi.nlm.nih.gov/pubmed/18476393.
20. Nasim M, Javeed A, Ashraf M, Omer M, Bukhsh A, Sharif A et al. Immunomodulatory activity of flurbiprofen in mice. Mortality. 2016; 10: 100.
21. Bharani SER, Asad M, Dhamanigi SS, Chandrakala GK. Immunomodulatory activity of methanolic extract of morusalba linn.(mulberry) leaves. Pak J Pharm Sci. 2010; 23 (1).
22. George A, Chinnappan S, Choudhary Y, Bommu P, Sridhar M. Immunomodulatory activity of an aqueous extract of polygonum minus huds on swiss albino mice using carbon clearance assay. Asian Pac J Trop Dis. 2014; 4: 398-400.
23. Huang JQ, Pang MR, Li GY, Wang N, Jin L, Zhang Y. Alleviation of cyclophosphamide-induced immunosuppression in mice by naturally acetylated hemicellulose from bamboo shavings. Food Agric Immunol. 2017; 28: 328-342. https://doi.org/10.1080/09540105.2016.1272553.
24. Hamdani DA, Javeed A, Ashraf M, Nazir J.

Evaluation of ketoprofen effects on humoral immunity and immune organs in mice. Pak J Zool. 2014; 46 (6).
25. Fulzele S, Satturwar P, Joshi S, Dorle A. Study of the immunomodulatory activity of haridradi ghrita in rats. Indian J Pharmacol. 2003; 35: 51-54.
26. Sudha P, Asdaq SM, Dhamingi SS, Chandrakala GK. Immunomodulatory activity of methanolic leaf extract of moringa oleifera in animals. Indian J Physiol Pharmacol. 2010; 54: 133-140. http://www.ncbi.nlm.nih.gov/pubmed/21090530.
27. Joung E-J, Gwon W-G, Shin T, Jung B-M, Choi J, Kim H-R. Anti-inflammatory action of the ethanolic extract from sargassum serratifolium on lipopolysaccharide-stimulated mouse peritoneal macrophages and identification of active components. J Appl Phycol. 2017; 29: 563-573.
28. Ray A, Dittel BN. Isolation of mouse peritoneal cavity cells. J Vis Exp. 2010; 35: e1488. http://www.ncbi.nlm.nih.gov/pubmed/20110936.
29. Geng Y, Xing L, Sun M, Su F. Immunomodulatory effects of sulfated polysaccharides of pine pollen on mouse macrophages. Int J Biol Macromol. 2016; 91: 846-855. http://www.ncbi.nlm.nih.gov/pubmed/27288698.
30. Allen IC. Delayed-type hypersensitivity models in mice. Mouse models of innate immunity: Springer; 2013. p. 101-107.
31. Liang L, Huang M, Xiao Y, Zen S, Lao M, Zou Y et al. Inhibitory effects of niclosamide on inflammation and migration of fibroblast-like synoviocytes from patients with rheumatoid arthritis. Inflamm Res. 2015; 64: 225-233. http://www.ncbi.nlm.nih.gov/pubmed/25708600.
32. Gately MK, Renzetti LM, Magram J, Stern AS, Adorini L, Gubler U et al. The interleukin-12/interleukin-12-receptor system: Role in normal and pathologic immune responses. Annu Rev Immunol. 1998; 16: 495-521. http://www.ncbi.nlm.nih.gov/pubmed/9597139.
33. Chen JR, Yang ZQ, Hu TJ, Yan ZT, Niu TX, Wang L et al. Immunomodulatory activity in vitro and in vivo of polysaccharide from potentilla anserina. Fitoterapia. 2010; 81: 1117-1124. http://www.ncbi.nlm.nih.gov/pubmed/20624446.
34. Frey BM. [mechanism of action of immunosuppressive agents]. Ther Umsch. 1993; 50: 71-76. http://www.ncbi.nlm.nih.gov/pubmed/8456418.
35. da Silva FM, Massart-Leen AM, Burvenich C. Development and maturation of neutrophils. Vet Q. 1994; 16: 220-225. http://www.ncbi.nlm.nih.gov/pubmed/7740747.
36. Shimozaki K, Nakajima K, Hirano T, Nagata S. Involvement of stat3 in the granulocyte colony-stimulating factor-induced differentiation of myeloid cells. J Biol Chem. 1997; 272: 25184-25189. http://www.ncbi.nlm.nih.gov/pubmed/9312131.
37. Witko-Sarsat V, Rieu P, Descamps-Latscha B, Lesavre P, Halbwachs-Mecarelli L. Neutrophils: Molecules, functions and pathophysiological aspects. Lab Invest. 2000; 80: 617-653. http://www.ncbi.nlm.nih.gov/pubmed/10830774.
38. Nguyen-Jackson H, Panopoulos AD, Zhang H, Li HS, Watowich SS. Stat3 controls the neutrophil migratory response to cxcr2 ligands by direct activation of g-csf–induced cxcr2 expression and via modulation of cxcr2 signal transduction. Blood. 2010; 115: 3354-3363.
39. Gordon S, Pluddemann A. Tissue macrophages: Heterogeneity and functions. BMC Biol. 2017; 15: 53. http://www.ncbi.nlm.nih.gov/pubmed/28662662.
40. Lehmann-Horn K, Kinzel S, Weber M. Deciphering the role of b cells in multiple sclerosis—towards specific targeting of pathogenic function. Int J Mol Sci. 2017; 18: 2048.
41. Yingjian L, Junming H, Min C, Chenyue L, Dachao Z, Yuanhua H et al. A health food high-peptide meal alleviates immunosuppression induced by hydrocortisone and cyclophosphamide in mice. Food Funct. 2013; 4: 1352-1359. http://www.ncbi.nlm.nih.gov/pubmed/23846393.
42. Nfambi J, Bbosa GS, Sembajwe LF, Gakunga J, Kasolo JN. Immunomodulatory activity of methanolic leaf extract of moringa oleifera in wistar albino rats. J Basic Clin Physiol Pharmacol. 2015; 26: 603-611. http://www.ncbi.nlm.nih.gov/pubmed/26103628.
43. Yang S, Tang X, Sheng X, Xing J, Zhan W. Analysis of the role of il-10 in the phagocytosis of migm(+) b lymphocytes in flounder (paralichthys olivaceus). Fish Shellfish Immunol. 2019; 92: 813-820. http://www.ncbi.nlm.nih.gov/pubmed/31271840.
44. de Lendonck LY, Eddahri F, Delmarcelle Y, Nguyen M, Leo O, Goriely S et al. Stat3 signaling induces the differentiation of human icos+ cd4 t cells helping b lymphocytes. PLoS One. 2013; 8: e71029.
45. Ye T, Xiong Y, Yan Y, Xia Y, Song X, Liu L et al. The anthelmintic drug niclosamide induces apoptosis, impairs metastasis and reduces immunosuppressive cells in breast cancer model. PLoS One. 2014; 9: e85887. http://www.ncbi.nlm.nih.gov/pubmed/24416452.
46. Braga CL, Felix NS, Teixeira DE, Vieira JB, Silva-Aguiar RP, Bose RM et al. Niclosamide attenuates lung vascular remodeling in experimental pulmonary arterial hypertension. Eur J Pharmacol. 2020; 887: 173438. http://www.ncbi.nlm.nih.gov/pubmed/32795515.
47. Sakkas LI, Bogdanos DP. Systemic sclerosis:

New evidence re-enforces the role of b cells. Autoimmun Rev. 2016; 15: 155-161. http://www.ncbi.nlm.nih.gov/pubmed/26497107.

48. Tabatabaei M, Liu Z, Finucane A, Parton R, Coote J. Protective immunity conferred by attenuated aroa derivatives of pasteurella multocida b:2 strains in a mouse model of hemorrhagic septicemia. Infect Immun. 2002; 70: 3355-3362. http://www.ncbi.nlm.nih.gov/pubmed/12065473.
49. Orth JH, Preuss I, Fester I, Schlosser A, Wilson BA, Aktories K. Pasteurella multocida toxin activation of heterotrimeric g proteins by deamidation. Proc Natl Acad Sci U S A. 2009; 106: 7179-7184. http://www.ncbi.nlm.nih.gov/pubmed/19369209.
50. Alam R, Fawzi EM, Alkhalf MI, Alansari WS, Aleya L, Abdel-Daim MM. Anti-inflammatory, immunomodulatory, and antioxidant activities of allicin, norfloxacin, or their combination against pasteurella multocida infection in male new zealand rabbits. Oxid Med Cell Longev. 2018; 2018.
51. Thatikonda S, Pooladanda V, Godugu C. Repurposing an old drug for new use: Niclosamide in psoriasis-like skin inflammation. J Cell Physiol. 2020; 235: 5270-5283. http://www.ncbi.nlm.nih.gov/pubmed/31846070.
52. Kelly A, Houston SA, Sherwood E, Casulli J, Travis MA. Regulation of innate and adaptive immunity by tgfβ. Advances in immunology. 134: Elsevier; 2017. p. 137-233.
53. Jang SG, Lee J, Hong SM, Song YS, Kim MJ, Kwok SK et al. Niclosamide suppresses the expansion of follicular helper t cells and alleviates disease severity in two murine models of lupus via stat3. J Transl Med. 2021; 19: 86. http://www.ncbi.nlm.nih.gov/pubmed/33632240.
54. Luwor RB, Baradaran B, Taylor LE, Iaria J, Nheu TV, Amiry N et al. Targeting stat3 and smad7 to restore tgf-beta cytostatic regulation of tumor cells in vitro and in vivo. Oncogene. 2013; 32, 19: 2433-2441. http://www.ncbi.nlm.nih.gov/pubmed/22751114.
55. de Ceuninck van Capelle C, Spit M, Ten Dijke P. Current perspectives on inhibitory smad7 in health and disease. Crit Rev Biochem Mol Biol. 2020; 55: 691-715. http://www.ncbi.nlm.nih.gov/pubmed/33081543.
56. Kuang C, Xiao Y, Liu X, Stringfield TM, Zhang S, Wang Z et al. In vivo disruption of tgf-beta signaling by smad7 leads to premalignant ductal lesions in the pancreas. Proc Natl Acad Sci U S A. 2006; 103: 1858-1863. http://www.ncbi.nlm.nih.gov/pubmed/16443684.
57. Li C, Iness A, Yoon J, Grider JR, Murthy KS, Kellum JM et al. Noncanonical stat3 activation regulates excess tgf-β1 and collagen i expression in muscle of stricturing crohn's disease. J Immunol. 2015; 194: 3422-3431.