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Silencing of IL-21 on HT29 and HCT116 Cells and Determining its Possible role in the Proliferation of Colorectal Cancer Associated with *Schistosoma Mansoni* Infection

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ABSTRACT

Background: Interleukin (IL)-21 is a cytokine produced by activated CD4 T-cells and natural killer T (NKT) cells. It is recognized for its anti-tumour effects and association with the development of autoimmune and inflammatory diseases. However, the role of IL-21 in the progression of pathogen-induced T-helper type 2 (Th2) responses, such as Schistosoma mansoni (S. mansoni) infection in colorectal tumourigenesis, remains unclear. **Objective:** The objective of this study was to determine the role of IL-21 in the proliferation of HT29 and HCT116 colorectal cancer cells after IL-21 is silenced. The proliferative effect of specific gene silencing may then correlate with colorectal cancer and S. mansoni infection. **Results:** The detection of IL-21 by ELISA and Western Blotting revealed that colorectal cancer patients infected with S. mansoni produced a high level of IL-21 in the serum compared to the serum of patients diagnosed with colorectal cancer only and the serum of patients infected with S. mansoni only. Moreover, the silencing of IL-21 in HT29 and HCT116 cells caused a significant reduction in the proliferation of cancer cells as determined by a Lactate Dehydrogenase (LDH) release assay. **Conclusion:** This study provides useful information indicating that IL-21 may be a potential target for the regulation, staging and surveillance of colorectal cancer caused by S. mansoni infection.

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INTRODUCTION

Modulation of the immune system is proposed as a novel approach to fight cancers. Cytokines are signalling molecules secreted by the immune system and represent a means to systemically modulate immune responses for the treatment of cancers (Rosenberg, 2001; Smyth *et al*., 2004). One of these cytokines is interleukin (IL)-21. This cytokine is produced by CD4 T-cells and natural killer T (NKT) cells (Parrish-Novak *et al*., 2000; Coquet *et al*., 2007). IL-21 targets a broad range of immune cells within both the lymphoid and myeloid lineage (Spolski and Leonard, 2008). This cytokine also increases anti-tumour activity in various mouse models (Leonard and Spolski, 2005), suggesting that IL-21 bridges the innate and adaptive immune responses (Collins *et al*., 2003). IL-21 impacts both the innate and adaptive immune responses due to its ability to act on multiple immune cells expressing the IL-21 receptor, such as B cells, NK cells, activated T-cells, dendritic cells (DCs), macrophages, fibroblasts and epithelial cells. IL-21 influences cell differentiation, cell fate, proliferation and the survival of diverse immune cell subsets (Kesselring *et al*., 2012). IL-21 also plays a prominent role in tumour growth and the immune surveillance of colitis-associated tumourigenesis. IL-21 controls the balance between T helper type 17 (Th17) and T helper type 1 (Th1) cell subsets and is necessary for the homeostasis of a tumour-supportive microenvironment characterized by extensive infiltration of Th17 cells. Secretion of this Th17 cell-associated cytokine leads to the induction of chemokines, matrix metalloproteinases and antimicrobial peptides in the surrounding tissue, leading to inflammation and the recruitment of neutrophils and macrophages that may lead to cancer development (Kesselring *et al*., 2012). Two independent and recent studies revealed a key role for this cytokine in promoting colitis-associated colorectal cancer (Stolfi *et al*., 2011; Jauch *et al*., 2011). Additional studies suggested that IL-21 is a Th2 cytokine that inhibits the differentiation of naive Th cells into IFN-γ–secreting Th1 cells (Wurster *et al*., 2002). Indeed, exogenous treatment with IL-21

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significantly inhibits IFN-γ production without affecting other Th1/Th2-associated cytokines, suggesting that the repression of IFN-γ by IL-21 is highly specific.

In *Schistosomiasis*, Th2 cytokines play a critical role in the pathogenesis of the disease (Pearce *et al*., 2002; Wynn, 2004). For example, IL-4/IL-13-, IL-4Ra- and Stat6-deficient mice all show significantly impaired granuloma formation and liver fibrosis following infection with *Schistosoma mansoni* (Chiaramonte *et al*., 1999; Fallon *et al*., 2000). Given the recent classification of IL-21 as a Th2 cytokine (Wurster *et al*., 2002; Mehta *et al*., 2005), the striking similarities between the IL-4 and IL-21 receptors (Habib *et al*., 2003; Sivakumar *et al*., 2004), and the critical role of the related IL-4Ra/Stat6 signalling pathway in this disease and other Th2 cytokine-driven inflammatory disorders (Wynn, 2003), a question evolving from these studies is whether IL-21R signalling plays a significant role in the initiation and/or maintenance of Th2 immunity. A study of *S. mansoni*-infected IL-21R-deficient and wild type (WT) mice (John *et al*., 2006) demonstrated that IL-21R deficiency had a profound effect on the progression of the disease in mice. Although the infection intensities were the same in WT and IL-21R-deficient mice, the egg-induced inflammatory response decreased significantly in the absence of IL-21R. A marked reduction in secondary granuloma formation and a faster resolution of primary granulomas in the lung were also observed. Together, these data illustrated a necessary role for IL-21R in granulomatous inflammation. Furthermore, the development of fibrosis significantly decreased in the IL-21R deficient mice, with a greater than 50% reduction in hepatic fibrosis by week 29 after infection.

The objectives of our present study were to determine the level of IL-21 in the serum of patients infected with *S. mansoni* and/or diagnosed with colorectal cancer to identify the role of IL-21 in colorectal cancer cells by performing specific gene silencing in the cancer cells and assessing the proliferation of the silenced cancer cells.

Methodology:

Study population, parasitological examination and serum samples collection:

We reviewed a total of 93 serum specimens from Sudanese patients infected with *S. mansoni* and/or diagnosed with colorectal cancer. All samples were collected from Khartoum hospitals (Khartoum Teaching Hospital, Niles Diagnostic Center and Hope Tower for Radiotherapy and Nuclear Medicine) in Sudan. The Tropical Medicine Research Institute (TMRI) - National Centre for Research (NCR) - Khartoum – Sudan approved this study. All patients underwent to stool examination using the Kato-Katz technique as described previously (Teesdale and Amin, 1976) to confirm the schistosomal infection. A 2 ml blood sample was collected from each patient in non-heparinized tubes. The samples were left to clot at room temperature and then centrifuged at 2,000 rpm for 15 min. The serum samples were stored at -70°C until use.

Extraction, separation and electro-transfer of proteins:

The proteins were extracted from the serum samples using Tri-reagent. The concentration of extracted proteins was adjusted to 20 µg/µL based on a protein assay. The extracted protein was separated by 10% reducing and discontinuous SDS polyacrylamide gel electrophoresis (SDS-PAGE), as described by Laemmli (1970), at 100 V for 2 h using a slab Mini-Gel system (Mini-V 8-10 Vertical Gel Electrophoresis Apparatus). The proteins in the serum samples were separated according to their molecular weights. The separated proteins were then transferred to a nitrocellulose paper (NCP) according to the method of Towbin *et al*. (1979) using a mini gel transfer unit. The protein transfer was performed at a constant voltage of 12 V for 30 min.

Western Blotting:

An enzyme immunoassay (EIA) was used to detect the proteins on the NPC. Each NCP was first washed with 0.05% PBS/T20 and blocked by incubating the NCP in 0.5% PBS-non-fat dry milk at room temperature. The NPCs were then incubated with polyclonal/monoclonal anti-human/mouse IL-21 antibody (Bio-legend Co.) at 1:250 dilutions in 0.05% PBS/T20 at 4°C overnight. Goat anti-mouse IgG conjugated with horseradish peroxidase enzyme (Bio-legend Co.) was added at a 1:3000 dilution in 0.05% PBS/T20 to the NCP and incubated at room temperature. The NPC was then soaked in 1:1 ECL substrate solution and the clear protein signals were developed on film. The films were left to dry and photographed using a scanner. The molecular weights of the developed protein bands were then calculated.

Detection of IL-21 levels using ELISA:

The levels of IL-21 in serum samples were measured using a Human IL-21 Sandwich ELISA Kit (eBioscience), according to the manufacturer's protocol. The level of IL-21 in each sample was estimated from the constructed standard curve.

Cell culture:

HT29 and HCT116 cells were obtained from the American Type Culture Collection, Manassas, VA. The cells were grown in DMEM medium (Dulbecco's Modified Eagle's Medium) supplemented with 10% FBS and 1% penicillin-streptomycin, and the cultures were incubated at 37°C in a 5% CO_2 incubator.

Silencing of IL-21 in HT29 and HCT116:

The HT29 and HCT116 cells were seeded in 6-well tissue culture plates at a density of 2×10^5 cells per well until the cells reached 60%-80% confluency. The cells were transfected with 4 µl of IL-21 siRNA duplex in 6 µl of transfection reagent (Santa Cruz Biotechnology, INC) and reduced serum medium (Santa Cruz Biotechnology, INC). The transfected cells were incubated for 5-7 h. The medium was then replaced with fresh normal growth medium, and the cells were grown for 24-72 h. The efficacy of silencing was detected using a fluorescent microscope after the cultures were incubated with a fluorescent conjugate control siRNA for 5-7 h at 37°C in a CO_2 incubator. The IL-21 level in the silenced cells was further confirmed by Western Blotting.

Assessment of HT29 and HCT116 proliferation:

The assessment of silenced cell proliferation was performed using the LDH Cytotoxicity Assay kit (BioVision), according to the manufacturer's instructions. Briefly, 100 µl of a reaction mixture (catalyst and dye solutions) was added to each well of transformed cells. The plate was incubated for 30 min at room temperature in the dark. The absorbance of each sample was then measured at 490 nm. A wavelength of 620 nm was used as the reference wavelength in this study. The percentage (%) of cytotoxicity or % of LDH released from the transformed HT29 and HCT116 cells was calculated.

Statistical analysis:

The data were analysed using SPSS statistical software (SPSS version 21) with a one-way ANOVA ordinary test and *t* test. A level of $p<0.05$ was considered statistically significant.

Results:

Population review:

In total, 93 serum samples were collected from patients recruited for this study, and the examinations of stool, histopathology and colonoscopy showed that 40 patients diagnosed with malignant colorectal cancer were also infected with *S. mansoni* (SCRC), whereas 26 patients were diagnosed with colorectal cancer (CRC) only and 27 patients were confirmed having *S. mansoni* infection only (SM).

Detection of IL-21 level:

The detection of the IL-21 level in the serum samples by ELISA showed that SCRC produced the highest level of IL-21 compared to CRC and SM, $P>0.01$. Western Blotting revealed bands for IL-21 in the SCRC, CRC and SM, and the IL-21 in the SCRC produced the greatest protein band intensity, as shown in Figure 1.

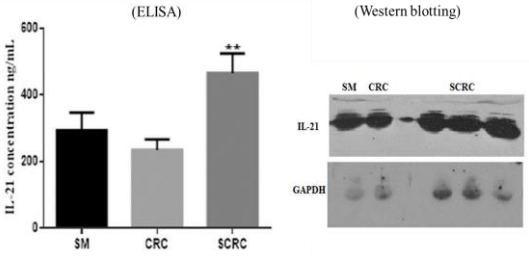

Fig. 1: The level of IL-21 in collected serum samples.

Silencing of the IL-21 gene:

The efficacy of IL-21 silencing in HT29 and HCT116 cell lines that was determined using Western Blotting revealed decreased IL-21 protein expression after specific gene silencing in the cells. The intensity of the protein bands in Figure 2 indicates that the IL-21 siRNA reduced the protein expression of IL-21 in HT29 and HCT116 cells compared to the control.

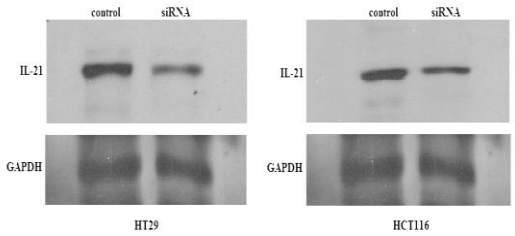

Fig. 2: The level of IL-21 protein expression in HT29 and HCT116 cells before (control) and after (IL-21 siRNA) IL-21 silencing. The protein band intensity was assessed using Western Blotting.

Proliferation assay:

The proliferative response of HT29 and HCT116 cells after IL-21 silencing that was measured using the LDH cytotoxicity assay detected the LDH released from damaged cells after IL-21 silencing at 24 h, 48 h and 72 h. Figure 3 demonstrates the increase in % cytotoxicity in HT29 and HCT116 after IL-21 silencing at specific time points, there was significant difference in proliferation between IL-21 siRNA silenced cell line and unsilenced in HT29 (p<0.001) and HCT116 (p<0.05).

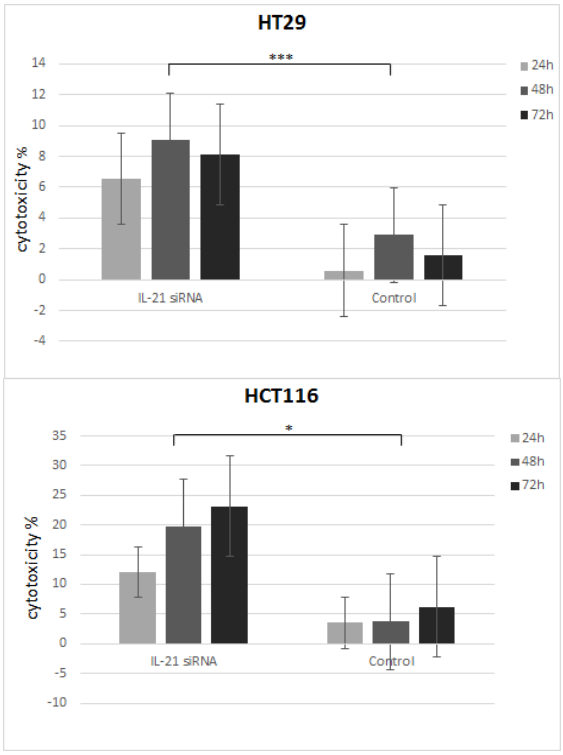

Fig. 3: The cytotoxicity of IL-21-silenced and unsilenced control HT29 and HCT116 colorectal cancer cells at 24 h, 48 h and 72 h.

Discussion:

Schistosomal colonic disease is a serious health problem in endemic areas. This condition may lead to complications, such as chronic intestinal *Schistosomiasis* and hepatosplenic *Schistosomiasis*, which has high morbidity and mortality if not diagnosed and treated early. At present, reports demonstrate that schistosomal colitis caused by *S. mansoni* is more commonly associated with the earlier onset of multicentric colorectal cancer (Emilio *et al*., 2007). Moreover, reports describe the association between bowel malignancy and *Schistosoma japonicum* (Chin-Sheng *et al*., 2010), and *Schistosoma hematobium* in urinary bladder malignancy (Pisani *et al*., 1997).

Of the 93 serum samples collected, our study found that 40 patients diagnosed with colorectal cancer were infected with *S. mansoni*. This phenomenon is consistent with previous studies in which there was a high rate of synchronous tumours in colorectal cancer patients infected with *Schistosomiasis* than patients diagnosed with spontaneous colorectal cancer only (Ming-Chai *et al*., 1980; Madbouly *et al*., 2007). This phenomenon can also be described in the context of colitis-associated cancer, suggesting the correlation of colorectal cancer with *S. mansoni* infection in the endemic area. Furthermore, *S. mansoni* infection is one of the primary reasons that hinder the development of developing countries.

The study of IL-21 in colorectal cancer and *S. mansoni* infection indicates that IL-21R deficiency has a profound effect on the progression of *S. mansoni* infection (John *et al*., 2006). The egg-induced inflammatory response decreased significantly in mice without IL-21R compared to wild-type. A marked reduction in secondary granuloma formation and a faster resolution of primary granulomas in the lung was also observed in the IL-21R deficient mice. This phenomenon demonstrates the importance of IL-21R in granulomatous inflammation. Another study by Kesselring *et al*. (2012) clearly showed that IL-21 had a prominent function in tumour growth and the immune surveillance of colitis-associated tumourigenesis.

Our present study reveals that patients diagnosed with colorectal cancer and infected with *S. mansoni* produce a higher level of IL-21 compared to patients infected with *S. mansoni* only and patients diagnosed

with colorectal cancer only. The study also found the role of IL-21 in the proliferation of HT29 and HCT116 colorectal cancer cells by silencing IL-21 gene expression using a specific IL-21 siRNA, suggesting the role of this cytokine in colorectal cancer cells. This result is consistent with a recent finding in colitis-associated colon cancer that indicated the absence of IL-21 induces apoptosis of tumour cells, activates the tumour immune surveillance and leads to limited tumour growth (Kesselring *et al*., 2012). By contrast, IL-21 induced tumour cell proliferation and limited the tumour immune surveillance, resulting in extensive tumour growth.

Conclusion:

This study demonstrates the proliferative role of IL-21 in colorectal cancer that may associate with *S. mansoni* infection. Silencing of the IL-21 gene reduces the proliferation of HT29 and HCT116 colorectal cancer cells. Thus, IL-21 is an attractive target for preventing and treating colorectal cancer associated with *S. mansoni* infection and may also be a target for novel drug development for these diseases.

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