IFN-γ and IL-18 in conditioned media of parasite-infected host and IL-21-silenced colorectal cancer cells

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Abstract. The presence of certain soluble factors may provide a possible selective advantage for a parasite to gradually modify cell proliferation in neighbouring cells, which may result in chronic diseases. These soluble factors present in the conditioned medium also allow the parasite to invade rapidly into more host cells. The present study aimed to determine the levels of a group of type 1 T helper (Th1) cytokines in the conditioned media of host cells infected with parasites and in IL-21-silenced colorectal cancer cells. The conditioned media of human foreskin fibroblasts (HFFs) parasitized with the RH and ME49 strains of *Toxoplasma gondii* for 10 days were prepared, and subsequently the levels of the Th1 cytokines in the conditioned media were determined by ELISA. HFFs were incubated with the growth media containing selected soluble factors, and cell proliferation markers were subsequently analysed by reverse transcription-quantitative PCR. The mRNA expression level of cell proliferation markers was also examined in IL-21-silenced HCT116 cells, where the levels of soluble factors in the conditioned media were also determined as aforementioned. The results of the present study demonstrated that HFFs parasitized with ME49 released elevated levels of IFN-γ and lower levels of IL-18 into the conditioned medium compared with the controls. These phenomena were not observed in the conditioned medium of HFFs parasitized with RH. Similar levels of these soluble factors were also detected in the conditioned medium of IL-21-silenced HCT116 cells. The results of the present study also revealed that Ki67 and proliferating cell nuclear antigen mRNA expression was altered in host cells incubated with various levels of IFN-γ and IL-18, as well as in IL-21-silenced HCT116 cells compared with the respective controls. In conclusion, the current study provided preliminary evidence on the fundamental molecular mechanisms of host-parasite interactions that result in chronic diseases, which may aid in the treatment of these diseases in the relevant endemic regions.

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Abbreviations: Th1, type 1 T helper; HFFs, human foreskin fibroblasts; siRNA, small interfering RNA; PCNA, proliferating cell nuclear antigen; cDNA, complementary DNA; OS, overall survival

Key words: interferon-γ, interleukin-18, host-parasite interaction, gene silencing, colorectal cancer, cell proliferation marker

Introduction

Toxoplasmosis is caused by the protozoan parasite *Toxoplasma gondii*, where >60% of populations are infected with these parasites (1,2). The infection often occurs in areas of lower altitudes, hot and humid climates (2). Our previous study demonstrated that a low dose of the virulent type I RH strain of *Toxoplasma gondii* (100 parasites) rapidly caused a lethal infection in mice within 4 days (3). By contrast, mortality with the avirulent type II ME49 strain of *T. gondii* occurred with a higher initial dose ($1x10^5$ parasites) at 6-8 days post-infection (3). The *T. gondii* strain RH exhibits high virulence in animals, but mice have been demonstrated to survive infection with the low virulence ME49 strain of *T. gondii* (4). It is hypothesised that parasitized host cells release soluble factors into the conditioned medium following *T. gondii* infection to regulate the parasite invasion into the host cells. However, the differences of these soluble factors in the conditioned media of host cells parasitized with RH and ME49 remain unclear. Different soluble factors are believed to be secreted into the conditioned medium by host cells parasitized with various strains of *T. gondii*, as these factors provide a possible selective advantage for the parasite to rapidly invade host cells (5). A previous study has demonstrated that the ability to develop chronicity of infection is dependent on type 1 T helper (Th1) cells (6). In addition, overstimulation of Th1 cytokines has been associated with the acute virulence of *T. gondii* (3). Therefore, soluble Th1 cytokines are likely to constitute the factors that underlie the differences between the conditioned media of host cells parasitized with RH and ME49.

The present study investigated the levels of soluble Th1 cytokines in the conditioned media of host cells parasitized with the RH and ME49 strains of *T. gondii* at different time intervals. The current study also compared the levels of soluble

factors in the conditioned media of IL-21-silenced colorectal cancer cells and the impact of the soluble factors on the mRNA expression level of cell proliferation markers in both the host cells post parasitic infection and colorectal cancer cells post silencing of the IL-21 gene. The IL-21 gene was silenced in a colorectal cancer cell line, as it has been indicated to reduce the proliferation of the cells, and it may also be used to study the molecular mechanisms of host-parasite interactions that cause chronic diseases with respect to colorectal cancer (7). The present study provided useful information on the fundamental molecular mechanisms of the host-parasite interactions that may aid in early diagnosis, novel prescription drugs and cost-effective strategies for the treatment of infectious disease-associated colorectal cancer in the future.

Materials and methods

Preparation of conditioned media of host cells parasitized with RH and ME49. The RH and ME49 strains of *T. gondii* were a kind gift from Prof. Rahmah Noordin, INFORMM, Universiti Sains Malaysia. The stocks were stored in liquid nitrogen. The parasites were cultured and propagated *in vitro* and adjusted to a concentration of 100 parasites/ml. The parasite numbers were estimated using a Neubauer haemocytometer chamber (Electron Microscopy Sciences, Inc.). Human foreskin fibroblasts (HFFs), which were originally purchased from the American Type Culture Collection (cat no. CRL-2522) and maintained in the laboratory, were used as the host cells and cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin (all from Thermo Fisher Scientific, Inc.). HFFs were used as the host for parasites since they are not differentiated, which allows the parasites to propagate rapidly (8). The growth medium of HFFs was changed every 2-3 days, and the cells were cultured at 37˚C in a humidified atmosphere with 5% CO_2. HFFs were seeded at a density of 1,000 cells/ml in T-75 cell culture flasks (Thermo Fisher Scientific, Inc.). The cell number was also estimated using a haemocytometer chamber. When HFFs were 80% confluent, RH or ME49 (100 parasites/ml) were added to the HFF feeder layer. The co-culture was incubated for 2-10 days. The growth medium was removed from the co-culture at different time intervals; every 2 days post-infection and used as the conditioned medium. The collected conditioned media, as well as the control media from non-parasitized HFFs (culture supernatants of HFFs only), were passed through a 0.22-mm filter (Thermo Fisher Scientific, Inc.) to separate the parasites, host cells and other cell debris. The conditioned media collected at various time intervals were used for the immunoassays of Th1 cytokines.

Immunoassay of Th1 cytokines in the conditioned media of parasite-infected host. Immunoassays for human IL-1β (cat no. KA0356), IL-10 (cat no. KA0125), IL-12p40 (cat no. KA0178), IL-18 (cat no. KA0561), IFN-γ (cat no. 3045) and TNF-α (cat no. P3453) were performed using commercially available ELISA kits (Abnova). According to the manufacturer's instructions, microtiter plates were pre-coated with antibodies specific for the Th1 cytokines. The collected conditioned media (~200 µl) were added to the respective wells and allowed to react with the bound antibody for 2.5 h at room temperature. The unbound substances were removed with a 1X PBS washing buffer, according to the manufacturer's instructions. An enzyme-linked antibody specific for each Th1 cytokine was added to the wells and incubated for 1 h at room temperature. Following another washing step, a substrate solution was added to the wells for colour development. The colour intensity was measured using a plate reader (Tecan Group, Ltd.) at 450 nm, and the colour development was proportional to the amount of the Th1 cytokines present in the samples. The cytokine level was calculated as the ratio of the experimental value (pg/ml) relative to the value in non-infected host cells. The statistically significant difference relative to day 2 ratio was considered.

Incubation of HFFs with media containing specific soluble factors. Briefly, HFFs were seeded at a density of 100 cells/ml into T-25 cell culture flasks (Thermo Fisher Scientific, Inc.) and maintained as aforementioned. When HFFs were ~80% confluent, the growth media were removed from the culture flasks. The HFF feeder layer was then exposed to growth medium containing 1 ng/ml human IFN-γ research grade (cat. no.: 130-096-873), 1 ng/ml Abnova human IL-18 recombinant protein (cat. no. P3632) or growth medium only for 4-10 days in triplicate (day 2 was excluded to reduce the use of cytokines and growth media). It is expected that an alteration in HFF proliferation was observed when the HFFs were incubated in growth media containing IFN-γ and IL-18 compared with growth medium alone. The incubated HFFs were subsequently harvested for analysis of mRNA expression, as described in the following section.

Analysis of the mRNA expression levels of cell proliferation markers in HFFs. Total RNA was extracted from HFFs incubated with specific soluble factors for 4-10 days using a commercially available RNeasy Mini Kit (Qiagen, Inc.) for total RNA extraction. RNA (1.0 µg) was reverse-transcribed into cDNA at 60˚C using a commercially available RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.), which was subsequently used for the analysis of cell proliferation markers at the mRNA expression level by quantitative PCR (qPCR). Primers for Ki67 and proliferating cell nuclear antigen (PCNA) were designed using Primer Express v2.0, and qPCR was performed using ABI 7500 Fast Sequence Detection System (both from Applied Biosystems; Thermo Fisher Scientific, Inc.). The primer sequences used were as follows: Ki67 forward, 5'-CCAACTGTTGGTCTC GCGTAAG-3' and reverse, 5'-ATCTGTCCAGCTGTAGTG CCCA-3'; PCNA forward, 5'-GGCACTCAAGGACCTCAT CAAC-3' and reverse, 5'-GTGAGCTGCACCAAAGAGACG T-3'; Transforming growth factor-α forward, 5'-CAGACC TTCCTACTTGGCCTGTAA-3' and reverse, 5'-GACGGA GTTCTTGACAGAGTTTTG-3'; Chemokine C-C motif ligand 5 forward, 5'-AGCCTCTCCCACAGGTACCAT-3' and reverse, 5'-GGCAGTAGCAATGAGGATGACA-3'; Epidermal growth factor forward, 5'-TGTGGTTCTCAGATT GGGCTATG-3' and reverse 5'-GATGAGGGCTTCAGCATG CT-3'; and GAPDH forward, 5'-CAAGTTCAACGGCACAGT CAAG-3' and reverse, 5'-CTCCTGGAAGATGGTGATTGG T-3'. GAPDH was used as the internal control. A total reaction volume of 25 µl was used, which included SYBR® Green

Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.), 900 nM of each primer and 5 μl cDNA (25 ng). The following thermocycling conditions were used: 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. Fold-changes in gene expression were calculated using the $2^{-\Delta\Delta Cq}$ method (9). The relative expression of each target gene in the samples of the host cells incubated with growth media containing soluble factors was normalised to that of the target gene in the samples of the host cells incubated with growth media alone (control). The expression level of each gene in fold-change was calculated relative to day 4 gene expression level.

Systematic review of the associations between IL-18, IFN-γ and IL-21. The following procedure was used for the systematic review: i) The research question 'what is the relationship between IL-18, IFN-γ and IL-21 in human diseases' was used as the topic of the analysis; ii) the systematic reference management software EndNote 9.0.1 (Clarivate Analytics) was used to extensively search the published studies on this topic worldwide; iii) the studies associated with this topic were screened in the PubMed databases at the National Library of Medicine (NLM; https://pubmed.ncbi.nlm.nih.gov/) to identify evidence for subsequent examination; iv) the studies on the associations between IL-18, IFN-γ and IL-21 in infectious disease and colorectal cancer research in an endemic region were extracted to answer the research question; v) the contents of each article were analysed, and the evidence was synthesised to determine bias with qualitative statistics tools, for example PCR and Immunoassays; and vi) the conclusion was returned into a report to support the analysis.

Silencing of the IL-21 gene in colorectal cancer cells. A mouse specific lyophilised IL-21 small interfering RNA (siRNA) reagent (cat. no. sc-39663; Santa Cruz Biotechnology, Inc.) was used to silence the IL-21 gene in HCT116 cells (American Type Culture Collection). The scrambled siRNA was included in the system. The cells were seeded in 6-well culture plates (Thermo Fisher Scientific, Inc.) at a density of $2x10^5$ cells/well in 2 ml antibiotic-free DMEM supplemented with 10% FBS. The cells were incubated at 37˚C in a humidified atmosphere of 5% CO_2 until they reached 80% confluency. Solution A was prepared by diluting 4 μl fluorescein-conjugated IL-21 siRNA duplex with 100 μl siRNA transfection medium (cat. no. sc-36868; Santa Cruz Biotechnology, Inc.). Solution B was prepared by diluting 6 μl of siRNA siRNA transfection reagent (cat. no. sc-29528; Santa Cruz Biotechnology, Inc.; as optimised in our previous study) with 100 μl siRNA transfection medium (7). Solution A was then directly added to Solution B and mixed gently by pipetting before mixing with 800 μl of siRNA transfection medium. The final mixture was incubated at room temperature for 15-45 min. The cells were washed with 2 ml siRNA transfection medium following the addition of the prepared mixture. The transfected cells were incubated at 37˚C for ≥72 h in a CO_2 incubator. The efficacy of gene silencing compared with that in cells transfected with scrambled siRNA was examined using a ZEISS Axio Observer Research Inverted Fluorescence Microscope (Carl Zeiss AG) with a magnification of x100. The IL-21-silenced cells were subjected to analysis of cell proliferation markers at the mRNA level, whereas the conditioned media collected from the IL-21-silenced cells were subjected to immunoassay of cytokines.

Analysis of cell proliferation markers at mRNA level and cytokines in conditioned media of IL-21-silenced cells by qPCR and ELISA. Total RNA was extracted from IL-21-silenced HCT116 cells at 1-3 days post-transfection. A total of 1.0 μg RNA was reverse-transcribed into cDNA, which was used for the analysis of cell proliferation marker expression at the mRNA level by qPCR. The same primers for the detection of Ki67 and PCNA were used as aforementioned, and the fold-changes in gene expression were calculated relative to those of the control cells (day 1), as aformentioned. On the other hand, the conditioned media collected from IL-21-silenced HCT116 cells at 1-3 days post-transfection were added to the wells and allowed to react with the bound antibodies on the microtiter plates that were pre-coated with antibodies specific for IL-18 and IFN-γ for 2.5 h at room temperature. The unbound substances were removed with a wash buffer, according to the manufacturer's instructions, and ELISA was subsequently performed as aforementioned. The cytokine level was calculated as the ratio of the experimental value in the silenced cells (pg/ml) relative to the value in control cells. Statistically significant differences were considered relative to day 1 ratio.

Statistical analysis. Data are presented as the mean ± SD. All statistical analyses were performed with one-way ANOVA followed by Tukey's post hoc test using GraphPad v6.01 software (GraphPad Software, Inc.). The experiments were repeated at least twice in three technical replicates to ensure reproducibility. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Th1 cytokine levels in the conditioned media of host cells parasitized with T. gondii. Investigation of the soluble factors IL-1β, IL-10, IL-12p40, IL-18, IFN-γ and TNF-α in the conditioned media of HFFs infected with strains of *T. gondii* at a range of time intervals revealed that host cells infected with the RH and ME49 strains of *T. gondii* released various levels of soluble factors into the conditioned media. The host cells parasitized with RH did not exhibit notable differences in the levels of soluble factors. IFN-γ was only significantly increased in the conditioned media compared with the control at day 8 of incubation (3.523-fold change; $P<0.05$; Fig. 1C). However, ME49-parasitized host cells released lower levels of IL-18 at day 8 (0.349-fold change; $P<0.05$) and day 10 (0.234-fold change; $P<0.05$) of incubation (Fig. 1D), and exhibited elevated levels of IFN-γ at day 8 (6.126-fold change; $P<0.01$) and day 10 (7.223-fold change; $P<0.01$) of incubation (Fig. 1C) in the conditioned media compared with those in the control cells. The levels of other soluble factors were either not detected (IL-12p40; TNF-α; data not shown) or exhibited no significant differences (IL-1β, Fig. 1A; IL-10, Fig. 1B) in the conditioned media of the host cells parasitized with RH or ME49.

mRNA expression levels of cell proliferation markers in IFN-γ- and IL-18-incubated HFFs. Determination of the

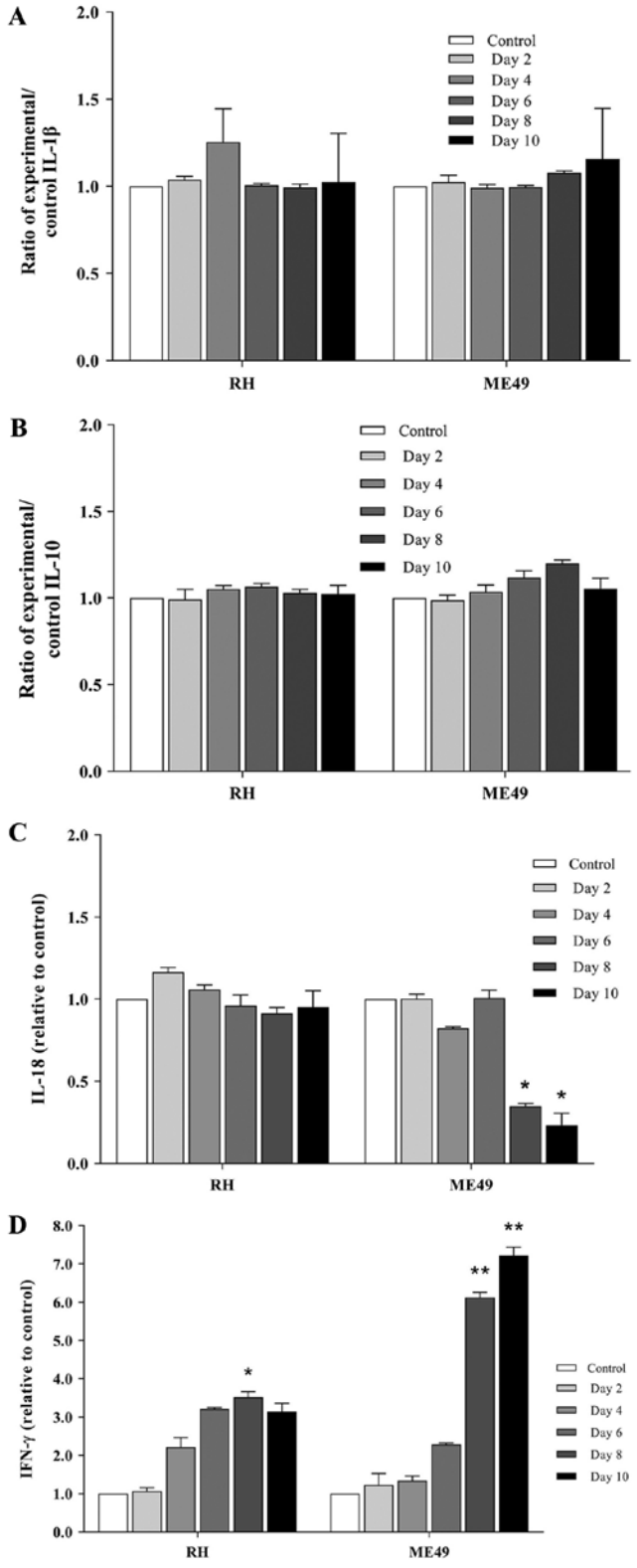

Figure 1. Cytokine levels in conditioned media of HFFs parasitized with RH and ME49 strains of *T. gondii*. (A) IL-1β, (B) IL-10, (C) IL-18 and (D) IFN-γ. The cytokine level was calculated as the ratio of the experimental value (pg/ml) relative to the value in non-infected host cells. At least two independent experiments were performed. Data are presented as the mean ± SD from triplicate cultures. *P<0.05 and **P<0.01 vs. day 2.

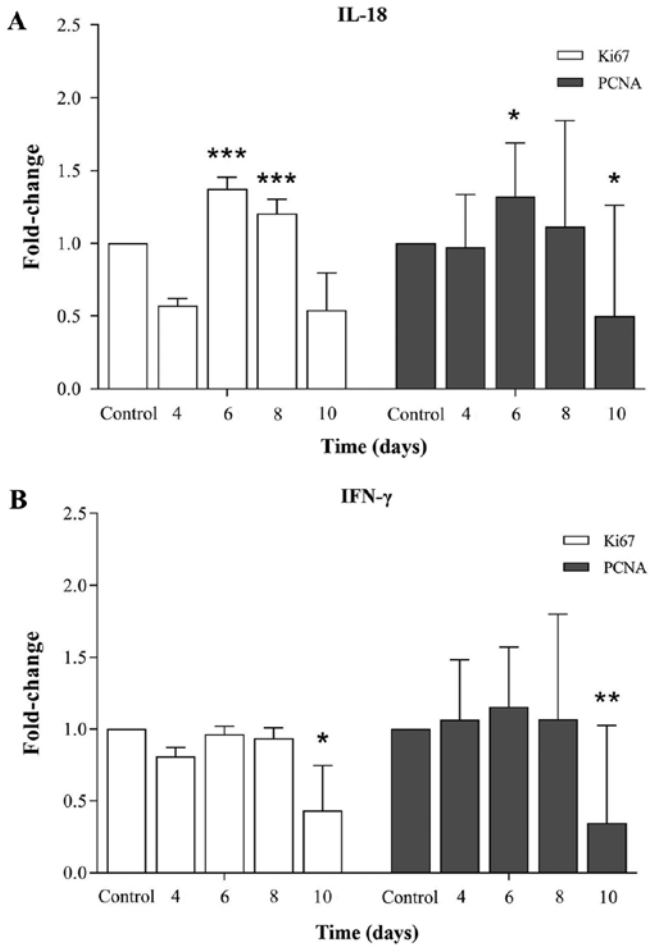

Figure 2. mRNA expression levels of cell proliferation markers in host cells incubated with growth media containing IL-18 and IFN-γ for different time intervals. (A) Ki67 and (B) PCNA. Host cells incubated with growth medium were used as a control. The target mRNA expression was normalised to the expression of GAPDH in the same sample. At least two independent experiments were performed. Data are presented as the mean ± SD from triplicate cultures. *P<0.05, **P<0.01 and ***P<0.001 vs. day 4. d, day; PCNA, proliferating cell nuclear antigen.

effects of IL-18 and IFN-γ on the mRNA expression levels of cell proliferation markers at different time intervals using reverse transcription (RT)-qPCR demonstrated that incubation of HFFs with growth media containing IL-18 resulted in a time-dependent bell-shaped curve in the mRNA expression levels of Ki67 and PCNA compared with the controls (day 4 gene expression level; Fig. 2A), whereas incubation with medium containing IFN-γ inhibited the mRNA expression of cell proliferation markers compared with the controls (day 4 gene expression level; Fig. 2B). In the growth media containing IL-18, the mRNA expression of Ki67 was increased at day 6 (1.376-fold change; P<0.001) and day 8 (1.204-fold change; P<0.001) of incubation, and it decreased to the initial level of mRNA expression at day 10 (0.539-fold change) of incubation. A similar level of mRNA expression was also revealed for PCNA in the IL-18-incubated host cells, where the mRNA expression of PCNA was increased to 1.321-fold change (P<0.05) and 1.114-fold change at days 6 and 8 of incubation, respectively, compared with the controls. The mRNA expression level of PCNA reached a 0.500-fold change (P<0.05) at day 10 of incubation. This pattern of alteration (bell-shaped curve) was not observed in the mRNA expression levels of the cell proliferation markers when the cells were incubated with growth media containing

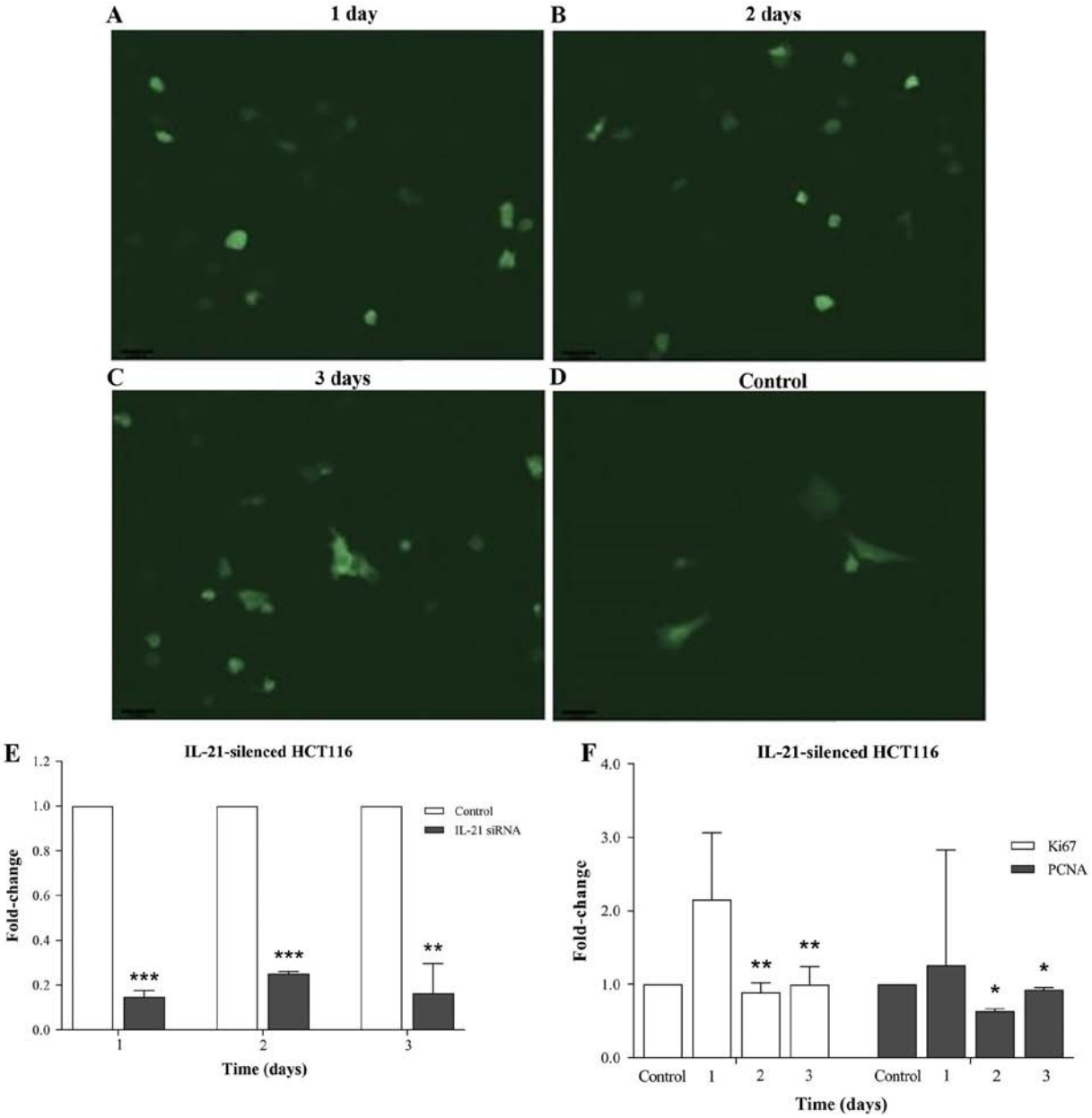

Figure 3. mRNA expression levels of cell proliferation markers in IL-21-silenced HCT116 cells. (A-D) Efficacy of IL-21 gene silencing for 1, 2 and 3 days under an inverted fluorescence microscope (magnification of x100 was used). The control image depicts the fluorescence of non-silenced cells. (E) Differences in IL-21 mRNA expression in cells transfected with IL-21 small interfering RNA and control (non-transfected) cells detected by reverse transcription-quantitative PCR. (F) mRNA expression was normalised to the expression of GAPDH in each sample. At least two independent experiments were performed. Data are presented as the mean ± SD from triplicate cultures. *P<0.05,**P<0.01 and ***P<0.001 vs. day 1. d, day; PCNA, proliferating cell nuclear antigen.

IFN-γ for different time intervals. The mRNA expression level of Ki67 and PCNA decreased to 0.434-fold (P<0.05) and 0.346-fold (P<0.01), respectively, at day 10 of incubation in the IFN-γ-incubated host cells compared with the controls. These results suggested that IL-18 was likely required for the host cell proliferation, whereas IFN-γ inhibited the host cell proliferation.

mRNA expression of cell proliferation markers in IL-21-silenced HCT116 cells. The images of IL-21-silenced HCT116 cells captured under an inverted fluorescence microscope are presented in Fig. 3A, and IL-21 mRNA expression differences in cells transfected with IL-21 siRNA and control (untransfected cells) were demonstrated using RT-qPCR (Fig. 3B). As the cells transfected with scrambled siRNA did not exhibit any significant differences compared with non-transfected cells in our previous study (7,10), non-transfected cells were used as control. Evaluation of the mRNA expression level of the cell proliferation markers Ki67 and PCNA using RT-qPCR demonstrated that the mRNA expression levels of Ki67 and PCNA were decreased in the IL-21-silenced HCT116 cells compared with those in the controls. The reduction in the mRNA levels of both markers was observed as early as day 1 following IL-21 silencing. The mRNA expression levels of Ki67 were significantly downregulated at days 2 (0.891-fold change; P<0.01) and 3 (0.993-fold change; P<0.01), and those

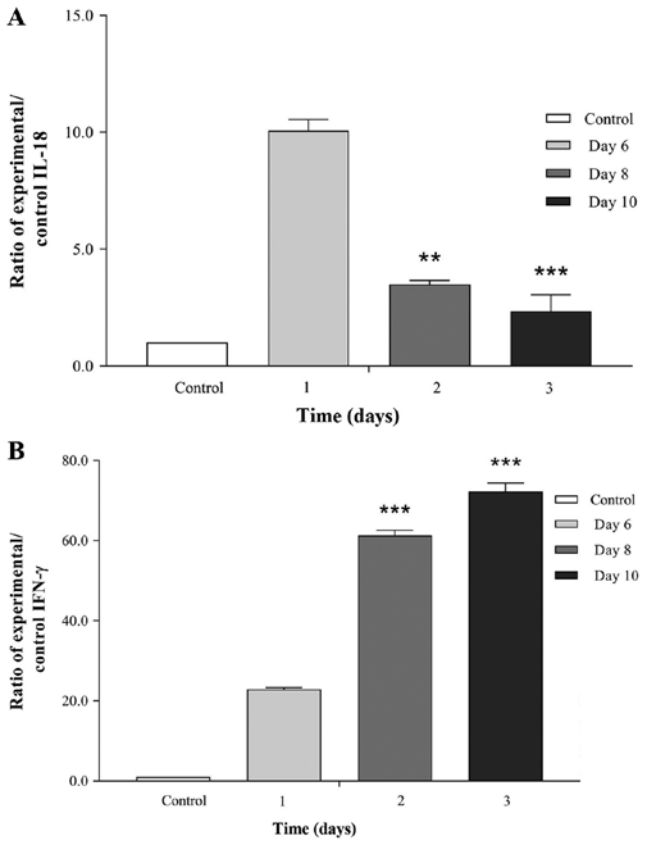

Figure 4. Soluble factor levels in conditioned media of IL-21-silenced HCT116 cells. (A) IL-18 and (B) IFN-γ. Conditioned media of non-parasitized host cells were used as a control. The cytokine level was calculated as the ratio of the experimental value (pg/ml) relative to that of the control. At least two independent experiments were performed. Data are presented as the mean ± SD from triplicate cultures. **P<0.01 and ***P<0.001 vs. day 1. d, day.

of PCNA were also significantly downregulated at days 2 (0.636-fold change; P<0.05) and 3 (0.924-fold change; P<0.05) following silencing of IL-21 in HCT116 compared to day 1 fold change (Fig. 3C). The mRNA expression level of other cell proliferation markers, such as transforming growth factor-α, chemokine C-C motif ligand 5 and *epidermal growth factor*, were not significantly altered in the IL-21-silenced HCT116 cells (data not shown).

IL-18 and IFN-γ levels in the conditioned media of IL-21-silenced HCT116 cells. The levels of the soluble factors IL-18 and IFN-γ in the conditioned media of IL-21-silenced HCT116 cells were analysed, and the results demonstrated that IL-21 silencing resulted in similar alterations of IL-18 and IFN-γ levels in the conditioned media of parasitized host cells. A reduction in IL-18 levels was observed on days 2 (3.459-fold change; P<0.01) and 3 (2.347-fold change; P<0.001) following IL-21 silencing compared with day 1 (10.070-fold change). By contrast, increased IFN-γ levels were observed on days 2 (61.260-fold change; P<0.001) and 3 (72.293-fold change; P<0.001) following IL-21 silencing compared with day 1 (Fig. 4A and B). Therefore, these results indicated that IL-18 and IFN-γ may be the soluble factors associated with the alterations in the levels of proliferation markers in IL-21-silenced HCT116 and parasite-infected host cells.

Discussion

The present study on Th1 immune responses following parasitic infection of host cells revealed that IL-18 and IFN-γ likely represented the growth factors that differentiated a possible selective advantage and disadvantage for RH and ME49 strains of *T. gondii* to invade into surrounding host cells. These factors may modify the proliferation of the host cells, which was observed as a reduction in Ki67 and PCNA mRNA expression levels in the IL-18- and IFN-γ-stimulated host cells at the end of the experiments. These soluble factors may also allow the parasites, such as ME49, to slowly invade into a higher number of surrounding host cells, resulting in chronic diseases. Similarly, reduction in the mRNA expression level of the cell proliferation markers Ki67 and PCNA was also observed in the IL-21-silenced HCT116 cells in the present study, which indicated that IL-21 may regulate the proliferation of diseased cells. Different levels of IL-18 and IFN-γ were also detected in the conditioned media of IL-21-silenced HCT116 cells. By studying HFFs (host cells) and HCT116 cells (diseased cells), the present study aimed to elucidate the fundamental molecular mechanisms of the host-parasite interactions that subsequently cause chronic diseases, such as colorectal cancer, and examine whether these two cell types released the same soluble factors in the conditioned media. The collective information may be useful for future treatment of infectious disease-associated colorectal cancer, and IL-21, IL-18 and IFN-γ may be used to develop tools for early diagnosis, novel prescription drugs and cost-effective strategies for the treatment of these diseases.

IL-18 and IFN-γ are Th1-type cytokines that are produced by the immune system in response to parasitic infection (11-13). Only host cells infected with ME49 were indicated to release reduced levels of IL-18 and elevated levels of IFN-γ in the conditioned media compared with the controls in the present study. It may be hypothesised that the ME49-parasitized host cells also secrete IL-21 into the conditioned media. IL-21 is a member of the common c chain family of cytokines, which includes IL-2, IL-4, IL-7 and IL-15 that has been reported to be involved in T-cell proliferation and homeostasis (14). A previous study has demonstrated that *IL-21r*$^{-/-}$ mice infected with *T. gondii* survived for ≥100 days post-infection; however, these mice displayed a defect in serum IgG production (15). IL-21 has been also associated with the differentiation of IL-10-producing CD4^{+} T cells, which have been reported to limit immune-mediated pathology during toxoplasmosis (16,17). IL-21-deficient mice chronically infected with *T. gondii* have been demonstrated to exhibit high numbers of parasites in the brain, which is associated with a decrease in parasite-specific antibody production and a marked reduction in the numbers of CD4^{+} and CD8^{+} effector T cells in the brain, resulting in diminished IFN-γ production (18). However, reductions in IFN-γ levels were not observed in the present study in the conditioned media of host cells parasitized with RH and ME49. By contrast, the levels of IFN-γ were elevated in the conditioned media of host cells parasitized with ME49 for ^{3}8 days, which suggested that the levels of IFN-γ production may be tissue-specific, and the alterations in the cytokine levels may provide a potential selective advantage for ME49 to cause chronic infection. However, this

Table I. Summary of the associations between IL-18 and IFN-γ with IL-21 observed in the present and previous studies.

	Cytokine			
Day	IL-18[a]	IFN-γ[a]	IL-21[b]	Comment
2	+	+	-	Early chronic parasitic infection
4	+	+	-	Early chronic parasitic infection
6	+	++	+	IFN-γ and cancerous factor levels start to be induced in host cells with chronic parasitic infection
8	-	+++	++	IFN-γ and cancerous factor levels continue to be induced when IL-18 is decreased in the conditioned media of host cells with chronic parasite infection
10	-	+++	+++	IFN-γ is maintained at a high level, and the cancerous factor continues to be induced when IL-18 is decreased in the conditioned media of host cells with chronic parasite infection

[a]Cytokines in chronic infection determined in the present study; [b]hypothesised cytokine levels based on a previous study (19). -, indicates low expression level, +, medium expression level; ++, high expression level; +++, very high expression level.

phenomenon was not observed in the type I parasitic infection with the RH strain.

A previous study has demonstrated that IL-18 and IL-21 in different combinations enhance IFN-γ production in human NK and T cells (19). The results of the present study revealed that infection with ME49 reduced the levels of IL-18, but increased those of IFN-γ in the conditioned media compared with those in the control cells. The overview of the association between IL-18 and IFN-g with IL-21 is summarised in Table I, whereby a low expression level of IL-21 is hypothesised at early chronic parasitic infection when both IL-18 and IFN-γ are maintained at moderate levels. When the infection time is prolonged, IL-21 continues to be induced, while IL-18 and IFN-γ are decreased and maintained at a high level, respectively. IL-18 is required for IFN-γ gene activation in both bacterial and viral infections (20-23). Therefore, a low level of IL-18 in the conditioned media of parasite-infected host cells may be insufficient to induce high levels of IFN-γ. The presence of other factors in the conditioned media, e.g. IL-21, likely contributes to high levels of IFN-γ and to the development of cancerous cells. This phenomenon may also explain the relationship between parasitic infection and colorectal cancer. IL-21 was the focus of the present study, as a high level of IL-21 expression has been detected in colorectal cancer cells, such as HT29 and HCT116 (7). In our previous study, a high level of IL-21 was also detected in the serum samples of patients diagnosed with colorectal cancer with a history of parasitic infections (24). IL-18 or IL-21 alone have been demonstrated to represent weak inducers of IFN-γ production, but the combination of IL-18 and IL-21 has been reported to induce notable activation of IFN-γ gene expression (19). Therefore, it was hypothesised that IL-21 is another soluble factor that may be present in the conditioned media, and it was examined in the present study whether IL-21 silencing resulted in the reduction of cell proliferation that may be associated with the levels of IL-18 and IFN-γ in the conditioned media.

In the present study, IL-18 and IFN-γ were demonstrated to alter the mRNA expression of the cell proliferation markers Ki67 and PCNA in IL-18- and IFN-γ-stimulated host cells, which was also observed in IL-21-silenced HCT116 cells. IL-18 is a uniquely pleiotropic member of the IL-1 family, and it is synthesised as a 24 kDa precursor protein and cleaved into an 18 kDa mature form by caspase-1 (25). The level of IL-18 was reduced in the conditioned media of host cells parasitized with ME49 for 8-10 days in the present study. Stimulation of the host cells with IL-18 induced an optimal level of cell proliferation markers dependent on the time following stimulation (bell shaped curve), which was reflected in the mRNA expression levels of Ki67 and PCNA. However, prolonged incubation of the host cells with IL-18 also reduced cell proliferation marker expression. These results were consistent with those of a previous study indicating that IL-18 exerted both cancer-promoting and cancer-suppressing functions (26). IL-18 has been revealed to promote the proliferation and invasion of pancreatic cancer cells, and higher IL-18 levels in pancreatic cancer tissues were associated with a shorter overall survival (OS), increased invasion and metastasis, compared with patients with lower IL-18 levels (26). However, in the same study, higher plasma levels of IL-18 were associated with longer OS. IL-18 has been demonstrated to exhibit antitumourtumour activity in preclinical models and increase the serum concentrations of IFN-γ, granulocyte macrophage colony-stimulating factor and soluble Fas ligand (27). By contrast, the role of IFN-γ was demonstrated to be more direct and straightforward in the present study. IFN-γ was induced in the conditioned media of the host cells parasitized with ME49, and stimulation of the host with IFN-γ reduced cell proliferation markers. These results suggested that IFN-γ may reduce cell proliferation markers, which was also observed in IL-21-silenced HCT116 cells in the present study. A previous study reported that intravesical instillations of 0.7 mg IFN-γ produced a significant cytostatic effect on superficial bladder cancer cells, which was evidenced by the decrease in growth fractions, measured using antigens of PCNA and Ki67 (28). IFN-γ has been also demonstrated to sustain the expression of PCNA and the G_1/S regulator retinoblastoma proteins, including cyclin D1, cyclin E and cdk2, and maintain low p27 levels (29). However, the effects

of prolonged IFN-γ stimulation on Ki67 and PCNA expression were only observed at day 10 in the current study. A more direct method, such as flow cytometry or western blotting, to evaluate the cell cycle-associated proteins should be utilised in future research to produce a stronger evidence for this hypothesis. The results of the analysis of the mRNA expression levels of cell proliferation markers in IL-21-silenced HCT116 cells indicated that IL-21 likely regulated the proliferation of HCT116 cells. The IL-21-silenced HCT116 cells may be used as a model to ensure whether the modification of diseased cell proliferation released the soluble factors as the aforementioned investigation outlines.

A previous study has revealed that IL-18-deficient mice were highly resistant to chronic *T. muris* infection, and *in vivo* treatment of normal mice with recombinant IL-18 suppressed IL-4 and IL-13 secretion. However, the treatment did not affect the level of IFN-γ in the mice (6). The present *in vitro* study did not observe an association between IL-18 and ME49 infection resistance. However, the levels of IL-18 and IFN-γ exhibited opposing trends during ME49 infection for 10 days, which supported the hypothesis that IL-18 does not function as an IFN-γ-inducing cytokine during chronic infections, but serves other roles, such as direct regulation of Th2 cytokines (30). Another study has demonstrated that $CD8^+$ T cells and IFN-γ were required for the host immunity to RH and ME49 (31). However, these differences may reflect stage-specific (tachyzoite vs. bradyzoite) or strain-specific (RH vs. ME49) requirements for the host immunity, which remain unclear, primarily since the majority of toxoplasma strains have been indicated to be virulent during secondary infections (32). Additional studies should be conducted in the future to provide stronger evidence for the validation of the hypothesis of the present study. To the best of our knowledge, *Schistosomiasis* attracts little attention and support worldwide owing to the geographical barriers and certain political issues (33). The disease may also be considered as unimportant as it primarily occurs in individuals living in poor, rural communities and in endemic regions (33). Moreover, current studies focus mainly on pandemic issues rather than neglected diseases.

In conclusion, the results of the present study may elucidate the fundamental molecular mechanisms of host-parasite interactions that cause chronic diseases. The results may also provide useful information for future studies on groups of genes that regulate Th cell responses during colorectal cancer and parasitic infection.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

PG, BYK and BY made substantial contributions to the design of the present study. CYO and AEA participated in all experiments under technical support provided by BYK. BYK interpreted the results, drafted and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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