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Original Research

Tetanus neurotoxin H_{CC} protein commits T cells to IFN-γ producing cells

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Abstract: A protective response against tetanus toxin and toxoid demands efficient specific T cell and B cell responses. Tetanus neurotoxin (TeNT), a 150 kDa polypeptide, is the main cause of tetanus disease. TeNT consists of two structurally distinct chains, a 50 kDa N-terminal light (L) and a 100 kDa C-terminal heavy (H) chain. C-terminal heavy (H) chain (fragment C) has two sub-domains named as proximal H_{CN} and carboxy sub-domain or H_{CC}. Beside neural binding property, H_{CC} has been recently found as an immunodominant module of TeNT. In the present study, we investigated the effects of recombinant H_{CC} (rH_{CC}) on the expression of lineage specific transcription factors and secretion of a panel of functional cytokines including IFN-γ, IL-4, and IL-17 from purified human T cells. Our results revealed that T-bet transcript level, as TH1 specific transcription factor, was significantly increased in the cells treated with 10 and 20 µg/ml of rH_{CC} following 48 h treatment($p<0.05$). Treated purified human T cells with rH_{CC} showed significant increase in IFN-γ mRNA level and cytokine secretion, but not IL-4 and IL-17, following 48 h treatment. In conclusion, our results showed that treatment of T cells with r H_{CC} resulted in development of Th1 lineage phenotype, which might lead to a specific and protective antibody mediated response against tetanus toxin.

Key words: H_{CC}, IFN-γ, T-bet, T cell; TeNT, Tetanus.

Introduction

Tetanus is a well-studied disease from the ancient times but remains one of the leading cause of death in developing countries. Almost, 800,000-1,000,000 deaths is caused by tetanus in these countries (1). Tetanus neurotoxin (TeNT), produced by the *Clostridium tetani*, is the major cause of disease symptoms (2). As first step of infection with *Clostridium tetani*, the toxin transmits from peripheral to central nervous system (CNS), which is directed by trans-synaptic connections. Subsequently, toxin vesicular endocytosis by inhibitory neurons leads to significant reduction of γ-amino butyric acid, a vital inhibitory neuro transmitter in CNS (3). Finally this sequence of toxin and CNS interactions culminates to the tetanus end stage or spastic paralysis.

TeNT is a ~ 150 kDa polypeptide which can be cleaved into two distinct chains by proteases. The 100 kDa C-terminal heavy chain (HC) and the 50 kDa N-terminal light chain (LC) bind by a disulfide bond to form active holotoxin (4). LC is known as a zinc metalloproteinase and functions as catalytic domain (5, 6). During disease course, LC blocks the γ-aminobutiric acid releasing by precisely cleaving the neuronal SNARE protein known as VAMP2 (vesicle associate membrane protein-2) (7, 8). Two different 50 kD fragments are characterized in the HC structure, N-terminal fragment (H_N) and C- terminal fragment (H_C) or fragment C. H_N is critical for LC penetration into neuronal membrane whereas fragment C functions as receptor binding domain and is completely non-toxic(2). Moreover, structural crystallography revealed that fragment C is consisted of N-terminal (H_{CN}) and C-terminal (H_{CC}) sub-domains. Further structural analysis showed that TeNT ganglioside binding property is mediated by H_{CC} sub-domain (9, 10).

Both humoral and cellular adaptive immunity systems can be triggered and developed by different parts of TeNT. In practice, the key to success or failure of vaccination is to select a proper target antigen enable to trigger both specific B and T cell responses. A number of studies focused on humoral immune responses and antibody production against different parts of TeNT, but less has been done on cell mediated immune mechanisms and responses against *Clostridium tetani* (11, 12). On the other hand, some of the studies have shown that fragment C is composed of "universal epitopes" with the capability of presenting by different human MHC class II molecules and activating specific $CD4^+$T cell responses (13, 14). Among different $CD4^+$T cell lineages, T helper 1 (TH1), as the major γ-IFN producer cells, has been found to be activated (15). Moreover, enumeration of interleukin-4 and interferon-γ-secreting human T cells reactivated with tetanus toxoid showed a drastic peak followed by a decline later (16). Howe-

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ver, the exact mechanism of H_{CC} interaction with cellular immunity components particularly with different functional CD4 T cell lineages e.i TH1, TH2 and TH17 remained to be discovered.

In the present study, we evaluated the effects of recombinant H_{CC} (rH_{CC}) on the mRNA level of $CD4^+$ T cell lineage specific transcription factors and secretion of a panel of functional cytokines including IFN-γ, IL-4, and IL-17 from purified human T cells.

Materials and Methods

Sampling

Heparinized peripheral blood was obtained from ten healthy adult volunteers. Peripheral blood mononuclear cells (PBMC) were isolated using Histopaque (Sigma, ST Louis, USA) density-gradient centrifugation, as described previously (17). PBMCs were washed twice with RPMI-1640 culture medium and resuspended in the medium supplemented with 10% fetal bovine serum, penicillin (100 IU) and streptomycin (100µg/ml). All of the human samples were collected upon Tabriz University of Medical Sciences review board approval and patient written informed consent.

T cells isolation by magnetic-activated cell sorting (MACS)

T cells were isolated in a negative selection manner by applying the magnetic activated cell sorting (MACS) and human $CD4^+$ T cell isolation kit II (Miltenyi Biotech, Bergisch-Gladbach, Germany), according to the manufacturer's instructions. In practice, negative selection resulted in the isolation of human $CD4^+$ T cells by means of non-$CD4^+$ T cells depletion. Briefly, a cocktail of biotin-conjugated monoclonal antibodies against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR γ/δ and glycophorin A were used for indirectly labeling of non-$CD4^+$ T cells that was accompanied by anti-biotin monoclonal antibodies conjugated MicroBeads as secondary labeling reagent . In a MACS® column the magnetically labeled non-$CD4^+$ T cells were trapped in the magnetic field of a MACS separator whereas the unlabeled $CD4^+$ T cells exit through the column.

T cells purity assessment

Purity of isolated T cells was confirmed by flow cytometry analysis with anti-CD3 PE and anti-CD4 FITC monoclonal antibodies (mAbs) (eBiosciences, San Diego, CA). Briefly, 10^6 purified cells were washed twice with washing buffer (PBS 0.15 M, 0.5%BSA, 0.1% NaN3) and resuspended in 100 µl of mentioned buffer. Subsequently, the cells were stained with anti-CD3 PE and anti-CD4 FITC mAbs and were incubated for 45 min at 4◦C in the dark. Finally, the cells were washed twice by wash buffer and scanned by flowcytometry (BD FACS Calibur). In all experiments the purity of the isolated $CD3^+/CD4^+$ cells were usually more than 95% (Figure 1).

Evaluation of T-bet, GATA-3 and RORγt mRNA levels using real time PCR

Purified CD4 T cells were incubated with three different concentrations of rH_{CC} (2, 10 and 20µg/ml), previously produced and purified by Yousefi et al (18). The mRNA levels of T-bet, GATA-3 and RORγt, as master transcription factors of TH1, TH2 and TH17 were determined by real time PCR. In brief, total RNA was extracted using Trizol (Sigma-Aldrich) from purified CD4 T cells treated with different concentrations of rH_{CC}. Synthesis of cDNA was carried out by applying 1 µg of total RNA and cDNA synthesis kit (Roche, Gipf-Oberfrick, Switzerland). Relative expression levels of T-bet, GATA-3 and RORγt transcripts were determined by the One-Step Quantitech SyberGreen Real Time PCR kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. In a parallel set of experiments, relative gene expression levels of IFN -γ, IL-4, and IL-17 were also evaluated. Primers for human T-bet,(F: 5'-GATGTTTGTGGACGTGGTCTTG-3' and R: 5'-CTTTCCACACTGCACCCACTT-3),human GATA-3(F: 5'-CTCCTCTCTGCTCTTCGCTACC-3' and R: 5'-GACTCTGCAATTCTGCGAGCC-3'), human RORγt (F: 5'-CCCACAGAGACAGCACC-GA-3'and R:5'-CCCACAGATTTTGCAAGGGA-3'), IFN–γ (F;5'-AGCTGACTAATTATTCGGTAACTG-3', R:5'- ATATTGCAGGCAGGACAACC-3'), IL-4(F: 5'-GCCTCACAGAGCAGAAGAACAC-3', R: 5'-GT-TGGCTTCCTTCACAGGACAG-3'), TNF-α(F:5'-GCCTCTTCTCCTTCCTGATCG-3' , R: 5'-GTTC-GAGAAGATGATCTGACTGC-3')and IL-17(F:5'-CCATCCCCAGTTGATTGGAA-3',R:5'CTCAGC AGCAGTAGCAGTGACA-3') were designed using primer blast software (NCBI, Primer Blast). Moreover, 18s rRNA (F: 5'-CCTGCGGCTTAATTTGACTCA-3', R:5'-AGCTATCAATCTGTCAATCCTGTCC-3'), was used as internal control. Differences in mRNA expression levels were calculated by ΔΔCt(target)=ΔCt(target, stimulated)−ΔCt(target, resting).

Evaluation of IFN-γ, IL-4 and IL-17 protein levels by ELISA

To evaluate the cytokine production from treated T cells, sandwich ELISA kits (R&D, Minneapolis, MN)

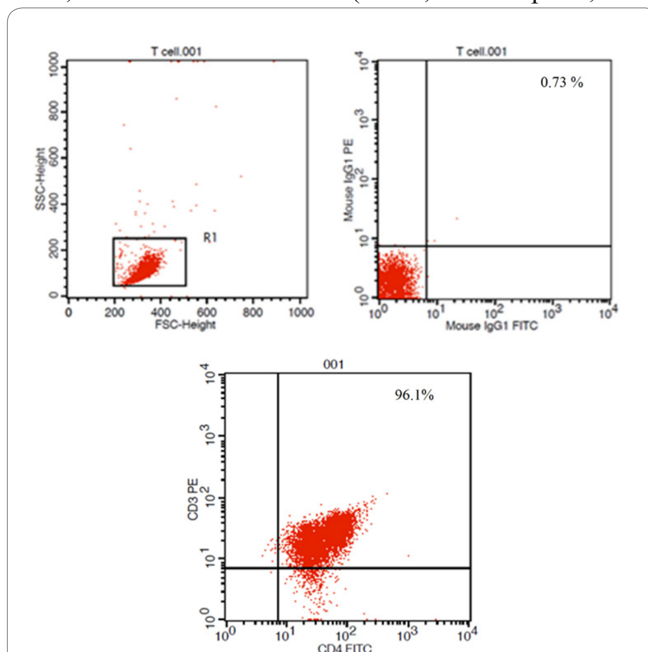

Figure 1. Purity of CD4 T cells isolated by MACS method. Two color staining was performed for evaluation of CD3 and CD4 expression levels on purified T cells. A) Unstained gated cells B) isotype control and C) CD3 and CD4 double positive T-cells.

were used for IFN-γ, IL-4 and IL-17 cytokines, according to manufacturer's instructions. Briefly, unconjugated, anti- IFN-γ capture Ab, anti-IL-4 capture Ab and anti-IL-17 capture Ab were employed for coating under condition of 100 mM Na2HPO4, pH 9.0 for 12 h at 4 ◦C in 96-well microtiter plates and the blocking were done with PBS containing 0.05% Tween 20 and 10% FBS. Cell free supernatants and cytokine standards, prepared in RPMI-1640 medium containing 10% FBS, were incubated for 2 h at room temperature. Detection of secreted cytokines was carried out using biotinylated mouse, anti-IFN-γ, anti-IL-4 and anti-IL-17 antibodies. Streptavidin alkaline phosphatase and *p*-nitrophenyl phosphate (4 mg/ml) were used as conjugate and substrate, respectively. The absorbance of each sample was measured at 405nm by microtiter plate reader. Cytokine concentrations in samples were quantified using the standard curves and regression analysis of mean absorbance.

Statistical analysis

For intergroup comparison *Kruskal-Wallis* non-parametric *ANOVA* test was performed. *P*-values below 0.05 were regarded as statistically significant.

Results

Purity of isolated T cells

At the first set of experiment, purity of the isolated cells by MACS was analyzed as CD3/CD4 subpopulation using flowcytometry. The results showed that more than >95% of isolated cells population was CD3/CD4 cells (Figure1).

Upregulation of the T-bet transcripts but not GATA-3 and ROR-γt in T cells treated with rH_{CC}

Real time analysis of T cell lineage specific transcription factors expression levels showed that T-bet transcript level was significantly increased in cells treated with 10(4.5 fold increases (±0.55 SD)) and 20 µg/ml (4.8 fold increases (±0.43 SD)) of rH_{CC} after 48 h treatment ($p<0.05$). However, there was not any significant differences in GATA-3 and ROR-γt transcript levels in treated T cells with different concentrations of rH_{CC} (Figure 2).

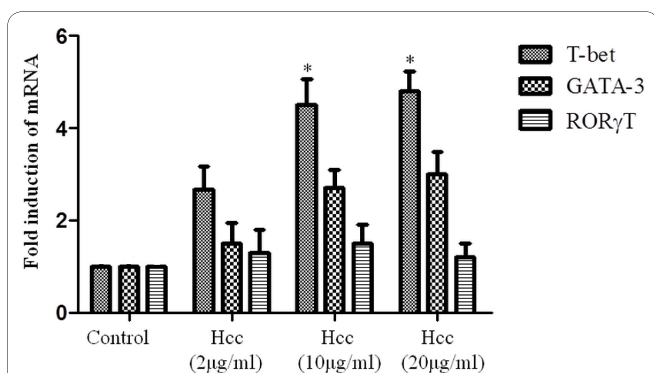

Figure 2. Relative mRNA expression levels of T-bet, GATA-3 and RORγt. Significant 4.5 and 4.8 fold increase in T-bet transcripts was observed after 48 h of T cell treatment by 10 and 20 µg/ml of rH_{CC}. Results shown are means from 10 samples/treatments. Values which are significantly different from control are indicated ($p < 0.05$).

Upregulation of the IFN-γ transcripts in the T cells treated with rH_{CC}

48 h treatment of purified T cells with 10µg/ml and 20µg/ml concentrations of rH_{CC} caused significant 5.67 fold (±0.66 SD) and 6 fold (± 0.5SD) increase at IFN-γ mRNA levels, respectively ($p<0.05$). However, no significant differences observed in IL-4, IL-17 mRNA levels in the treated cells in comparison with untreated cells (Figure 3).

rHcc induces IFN-γ production from purified T cells

Cytokine secretion of T cells treated with different concentrations of rH_{CC} was measured following 48 hours using indirect ELISA. The results demonstrated that rH_{CC} in 10µg/ml and 20µg/ml concentration caused significant increases of IFN-γ production. Levels of IFNγ formation increased from 97.61 (± 31.4 SD) pg/ml to now 241.6 (±53.39 SD) and383.9 (±26.15 SD) in 10µg/ml and 20µg/ml concentration of rH_{CC} (Figure 4A, $p<0.05$). However, the results revealed that rHcc could not stimulate T cells to produce IL-4 and IL-17 at any concentrations (Figure 4B and 4C).

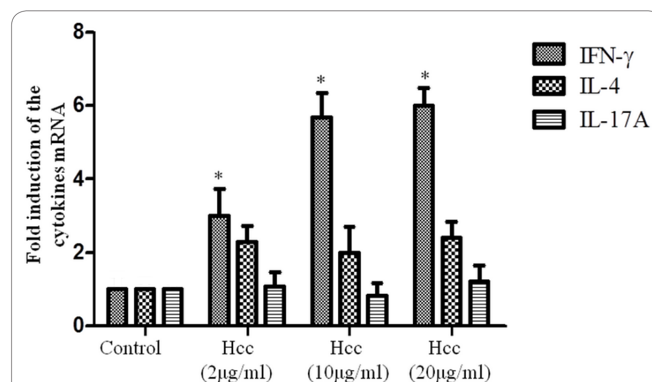

Figure 3. Relative mRNA expression levels of IFN-γ, IL-4 and IL-17. mRNA level of IFN-γ, IL-4 and IL-17 was analyzed in 48h rH_{CC} treated T cells using real time PCR. Amplification of PCR was normalized against 18srRNA. The transcript level of IFN-γ was significantly increased in T cells treated with different concentrations of rH_{CC} * represents $p<0.05$.

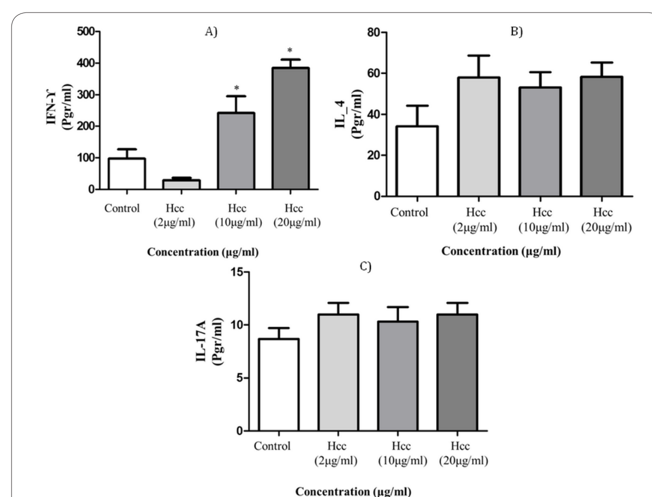

Figure 4. Cytokine production of treated T cells with different concentrations of rH_{CC}. A) IFN-γ secretion level in rH_{CC} treated T cells, B) secretion level of IL-4 and C) secretion level of IL-17 after 48 h in T cells treated with different concentrations of rH_{CC} (2, 10, 20µg/ml) using indirect ELISA. .* represents $p<0.05$.

Discussion

The certain goal of vaccination is to generate a protective immune response against disease caused by microorganisms or antigens. Neutralizing antibody mediated immunity against toxin of certain bacteria demands a subtle collaboration of specific B and T cell interaction. In practice, several functional and in silico studies carried out to meet this demand.

In this study we explored the effects of recombinant H_{CC} on the lineage specific T cells responses. rH_{CC} was produced and characterized precisely in our previous studies (18). At the first set of experiments, CD4 T cells were purified and treated with different concentrations of rH_{CC}. The mRNA expression levels of T-bet, GATA-3 and ROR-γt as master transcription factors of TCD4+ TH1, TH2 and TH17 subsets, showed that T-bet, but not GATA-3 and ROR-γt, was significantly up-regulated. In accordance with up-regulation of TH1 specific transcription factor, a significant up-regulation of IFN-γ m-RNA was also observed in the treated cells. T cells cytokine secretion levels were assessed by sandwich ELISA. The results confirmed the real time PCR findings, regarding induction of IFN-γ, but not IL-4 and IL-17 production and secretion in the rH_{CC} treated purified T cells. The latter data also indicates that rH_{CC} primes T cells to develop functional TH1 IFN-γ producing cells.

A large body of evidences showed that the whole toxoid of tetanus has the capability of inducing clonal T cell activation. Parronchi et al, showed that the majority of tetanus toxoid specific T cells are $CD4^+$ lineage with high IFN-γ secretion capability (15). TH1 associated cytokines, particularly IFN-γ, generally involved in class switching of B cells which are the pivotal effector cells for establishing protective immune response against tetanus. In our previous study, we found that anti-fragment C antibodies can inhibit toxin-receptor interaction in a dose dependent manner (18). Furthermore in a recent study, we revealed that seven out of the eleven tetanus toxin specific monoclonal antibodies, were reactive to fragment C (11). During humoral immune responses against tetanus toxin, fragment C is recognized as an immunodominant module of the toxin. In accordance, we also demonstrated that six out of seven anti fragment C antibodies inhibited the interaction of TeNT and it's receptor in a dose dependent manner (18).T cell activation, particularly TH1 subset, can develop a cytokine network which is essential for B cell class switching and generation of specific IgGs, leading to a protective immunity against tetanus. As previously mentioned, fragment C and its H_{CC} sub-domain contains 'universal epitopes' which can be processed and presented with a variety of human MHC class II molecules (13, 14) . This enables these epitopes to activate different T cell populations including TH1 IFN-γ producing cells. Interestingly our preliminary data provide evidence that rH_{CC} commits T cells preferentially to TH1 IFN-γ producing cells.

One explanation for the mechanism in which rH_{CC} stimulated purified human T cells isolated by MACS is the presence of contaminating antigen-presenting cells (APC)/other accessory cells in T cells fraction. Lancioni et al. (19) showed that isolation of T-cells using the MACS method results in contamination by other immune cell subsets such as $CD14^+$ monocytes, NK cells, plasmacytoid and myeloid dendritic cells, $CD8^+$ T-cells, and B-cells. Contaminating antigen-presenting cells processed and presented rH_{CC} to co-harvested T cells and stimulated isolated and purified T cells.

Taking our findings into accounts, rH_{CC} might be a proper antigenic candidate to be employed in new generation of tetanus vaccine and monoclonal antibody. Further studies are required to prove H_{CC}'s adjuvant capacity and the possibility of rH_{CC} application as adjuvant in new generation of vaccines.

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