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Response of Human T Cells to Tetanus Neurotoxin H_{CC} Sub-Domain: T Cell Cytokine Production and Activation Marker Induced by H_{CC}

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ABSTRACT

Tetanus is caused by the tetanus neurotoxin (TeNT), a 150 kDa single polypeptide molecule which is cleaved into active two-chain molecules composed of a 50 kDa N-terminal light (L) and a 100 kDa C-terminal heavy (H) chains. Fragment C is further subdivided into two subdomains: the proximal H_{CN} subdomain and the extreme carboxy subdomain, H_{CC}. H_{CC} is considered as an immunodominant part of TeNT and is responsible for TeNT binding activity to neurons.

In the present study, we investigated the ability of recombinant H_{CC}(r H_{CC}) to induce T cell activation. Our results showed that recombinant H_{CC} has a stimulatory effect on IFN-γ secretion by T cells after 48h co-incubation in the presence of anti-TLR-2 Ab. Also, Hcc can induce the expression of CD69 on T cells.

Our finding indicated that stimulatory effects of H_{CC} on T cells are TLR-2 independent and anti-TLR-2 inhibitory antibody fails to neutralize H_{CC} stimulatory effects on T cells.

Furthermore, H_{CC} is critical for immunogenic activity of TeNT and is able to induce T cells through TLR-2 independent pathway.

Keyword: H_{CC} subdomain; TeNT; T cell; TLR-2; IFN-γ

INTRODUCTION

Tetanus is a life threatening and highly fatal infectious disease among human and domestic animals.[1] World Health Organization estimated approximately 1000000 deaths from tetanus annually worldwide despite the fact that tetanus is now a rare disease in developed countries. In spite of having an efficient vaccine using tetanus toxoid, tetanus remains an important cause of death in developing countries including 580000 deaths from neonatal tetanus, with 210000 in South East Asia and 152000 in Africa. Tetanus neurotoxin (TeNT) is produced by *Clostridium tetani* which is a natural habitat of soil, but can also be isolated from the stool samples of domestic animals and humans.[1,2] Tetanus neurotoxin (TeNT) is synthesized as a single-chain polypeptide of 150 kDa

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and is subsequently cleaved to generate an active di-chain toxin, in which the light (LC) and heavy chains (HC) are linked by a single disulphide bond. The LC acts as a zinc-dependent endopeptidase and inhibits neurotransmission through cleavage of soluble NSF attachment protein (SNAP) Receptor (SNARE) protein.[3-5] The HC mainly involves in TeNT binding to Gangliosides of the 1b series (GT1b) ganglioside on the surface of neurons and is composed of two functionally distinct domains, the N-terminal half (HN: 50 kDa) and the carboxy terminal half (HC or fragment C: 50 kDa).[6] Fragment C is further subdivided into two subdomains: the proximal H_{CN} subdomain and the extreme carboxy subdomain, H_{CC}. This latter subdomain (H_{CC}) holds the key amino acid residues responsible for the binding activity of the clostridial neurotoxins (CNTs).[7] Some studies have shown that tetanus toxin fragment C contains "universal epitopes" for human $CD4^+$ T lymphocytes which are easily bound to a variety of human MHC class II molecules. Therefore, This property enables "universal epitopes" to sensitize $CD4^+$ T lymphocytes of most or all individuals.[8,9] It has been demonstrated that tetanus toxin specific T cells are mainly $CD4^+$ cells secreting Th1 cytokines such as IFN-γ.[10] Toll-like receptors (TLRs) as type I integral membrane glycoproteins play a central role in response to various pathogen-associated molecular patterns (PAMPs) derived from bacteria, fungi, viruses and protozoa.TLR2 can recognize wide variety of bacterial cell wall components such as peptidoglycan,lipoarabinomannan, diacyl or triacyllipopeptides, mycobacteria and mycoplasma.[11] Upon binding to these ligands, TLR2 induces inflammatory responses and cytokines production especially IFN-γ and TNF-α.[12]

We previously produced recombinant H_{CC} (rH_{CC}) fragment of TeNT.[13] In the present study, we investigated the ability of rH_{CC} to induce T cells for producing interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) and the expression of activation marker (CD69) on T cells. The effect of rH_{CC} was also investigated in the presence and absence of anti-TLR-2 inhibitory antibodies to show the mechanism by which rH_{CC} activates T cells.

MATERIALS AND METHODS

T Cells Isolation by Magnetic-activated Cell Sorting (MACS)

Blood was obtained from 10 healthy donors and peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood on a Ficoll gradient, as described previously.[14] Human $CD4^+$ T cells were isolated by depletion of non-$CD4^+$ T cells (negative selection) using the magnetic affinity cell sorting (MACS) Human $CD4^+$ T cell isolation kit II (Miltenyi Biotech, Bergisch-Gladbach, Germany), according to the manufacturer's instructions.. Non-$CD4^+$ T cells were magnetically labeled with a cocktail of biotin-conjugated monoclonal antibodies against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR γ/δ and glycophorin A and anti-biotin monoclonal antibodies conjugated to MicroBeads, as secondary labeling reagent. The magnetically labeled non-$CD4^+$ T cells were depleted by retaining them on a MACS® column in the magnetic field of a MACS separator (MiltenyiBiotec, BergischGladbach, Germany), while the unlabeled T helper cells were passed through the column.

T Cell Activation Assessment

Firstly, T cells purity was evaluated by flow cytometry using anti-CD3 FITC (eBiosciences, San Diego, CA) monoclonal antibodies (mAbs). Briefly, after twice washings with washing buffer (PBS 0.15 M, 0.5%BSA, 0.1% NaN3), 1×10^6 cells were resuspended in 100 µl washing buffer and stained with appropriate anti-CD3 FITC mAb and incubated for 45 min at 4◦C in the dark. Subsequently, the cells were washed twice with wash buffer and scanned by flow cytometer (BD FACSCalibur). Purity> 90% of the isolated CD3+ cells was obtained in all experiments (Figure 1).

Secondly, for assessment of the activation of T cells that were incubated with three different concentrations of rH_{CC} in the presence and absence of human anti-TLR-2 monoclonal antibody (MAb-hTLR2) (Invivogen, San Diego, CA), we analyzed the expression of CD3 and CD69 by flow cytometry using anti-CD3 FITC and anti-CD69 PE (eBiosciences, San Diego, CA). Finally data analysis was performed using theFlow Josoftware(Tree Star Inc., USA).

Co-incubation of Purified T Cells with rH_{CC} and Anti-TLR2 Antibody

Recombinant H_{CC} was produced by Yousefi et al. as described previously.[13] 1×10^6purified T cells were incubated with three different concentrations of H_{CC} (2, 10 and 20 µg/ml) in the presence and absence of anti-TLR-2 blocking antibody (invivogen, San Diego, CA) in 1 ml RPMI-1640 medium supplemented with 10% FBS for 48 h at 37 ◦C, 5% CO2.

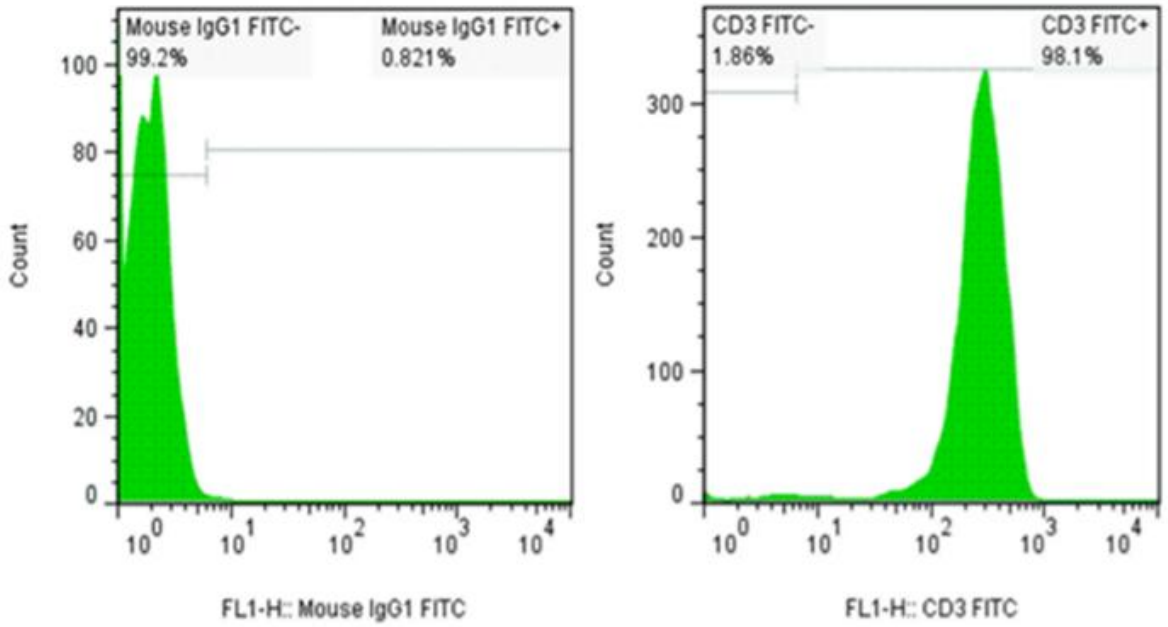

Figure1. The purity of CD3$^+$ T cells isolated from PBMC using MACS technique. The separated T cells were stained with FITC anti-CD3 and isotype control antibody. isotype controlled cells (left) and anti CD3 stained T cells (right) of enriched fraction are presented.

Determination of IFN-γ, TNF-α and IL-4 Secretion by ELISA

To investigate the effect of rH$_{CC}$ on cytokine production, cell-free culture supernatants of treated and control T cells were harvested and the concentrations of IFN-γ, TNF-α and IL-4 were determined by standard sandwich ELISA kits (R&D, Minneapolis, MN) according to the manufacturer's instructions. In brief, 96-well microtiter plates were coated with an unconjugated anti- TNF-α capture mAb, anti- IFN-γ capture mAb or anti-IL-4 capture mAb in 100mM Na2HPO4, pH 9.0 for 12 h at 4 ◦C, and then the plates were blocked using PBS containing 0.05% Tween 20 and 10% FBS. Cell supernatants and recombinant TNF-α, IFN-γ and IL-4 standards (R&D Systems) were cultured in RPMI-1640 medium supplemented with 10% FBS for 2 h at room temperature. Bound TNF-α, IFN-γ and IL_4 were detected using a biotinylated mouse anti-TNF-α, anti-IFN-γ and anti-IL-4 Ab in 1% BSA for 1 h. The plates were developed using streptavidin alkaline phosphatase conjugate with *p*-nitrophenyl phosphate (4 mg/ml) as substrate. The absorbance at 405 nm was read using a microtiter plate reader, and the concentrations of so-called cytokines were calculated from a standard curve of recombinant human TNF-α,IFN-γ and IL-4.Cytokine concentrations of each sample were calculated by regression analysis using the mean absorbance (average of triplicate readings of the sample added).

Statistical Analysis

For the cytokine production analysis, an intergroup comparison was performed by *Kruskal-Wallis* non-parametric *ANOVA* test. *P*-values below 0.05 were regarded as statistically significant.

RESULTS

rH$_{CC}$ Induced CD69 on T Cells

The levels of CD3 and CD69 surface markers on T cells treated with rH$_{CC}$ in the presence or absence of anti-TLR2 blocking antibody were measured using flow cytometry. In comparing with control group, rH$_{CC}$ induced CD69 expression on T cells. This effect of rH$_{CC}$ was also observed in the presence of anti-TLR-2 blocking antibody (Figure 2). The overall percentages of CD3^{bright} and CD69^{bright} T cells co-incubated with 10 µgr/ml rH$_{CC}$ in the presence and absence of anti TLR-2 inhibitory antibodies (P<0.05) showed significant up-regulation of CD69 expression on T cells (P<0.05) (Figures 2 and 3).

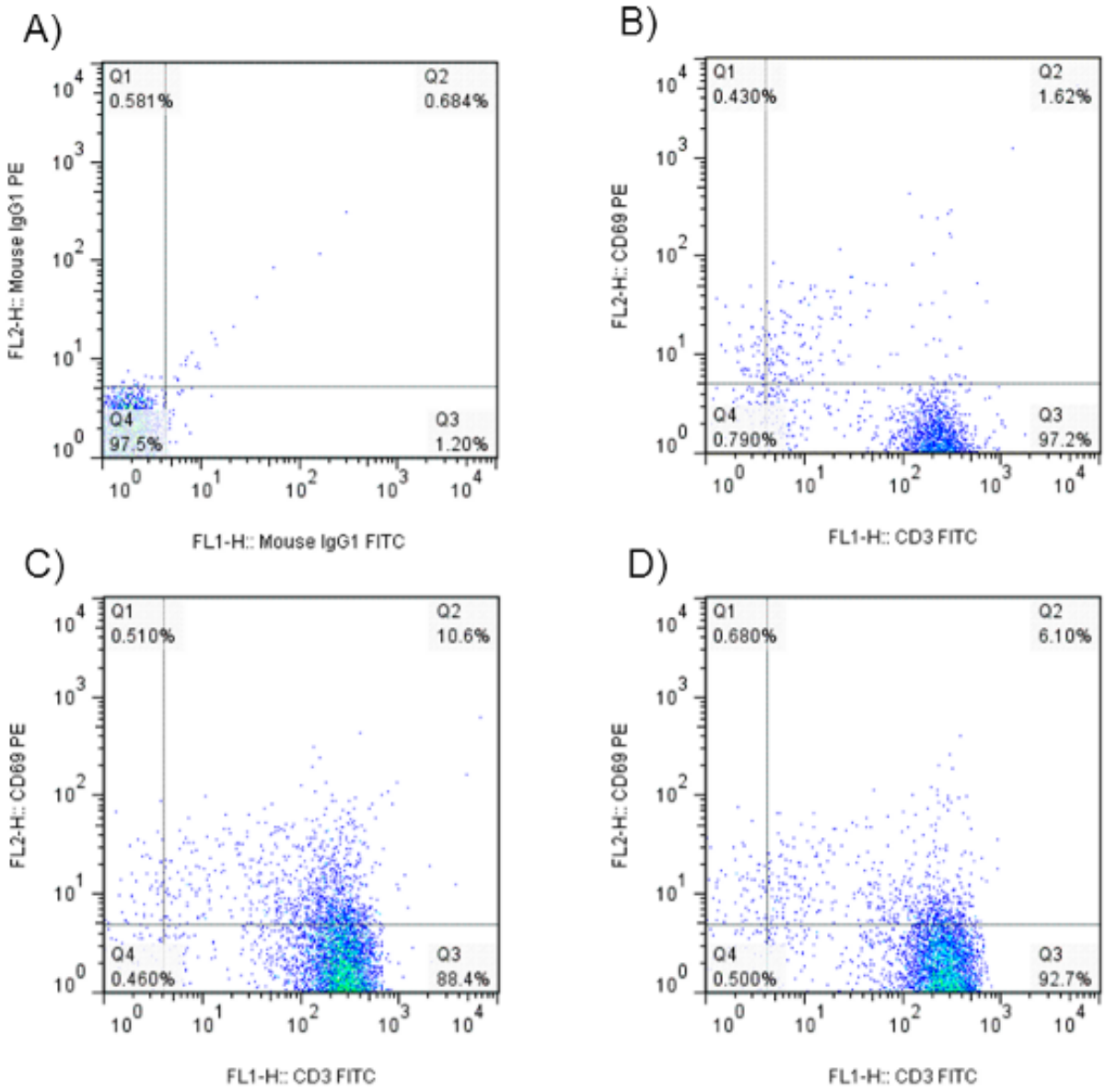

Figure 2. Representative results showing the effects of rH_{CC} on the expression of T cell activation marker. Expression levels of T cell activation marker (CD69) were measured on T cells by two color staining method (anti-CD3 FITC and anti-CD69 PE). A) Isotype control B) Untreated cells C) Purified T cells treated with 10 µgr/ml rH_{CC}. D) Purified T cells co-incubated with rH_{CC} and anti TLR-2 blocking antibody.

rH_{CC} Induced IFN-γ Production in T Cells in the Presence of Anti-TLR-2 Blocking Antibody

The cytokine analysis revealed that rH_{CC} had a stimulatory effect on IFN-γ secretion of T cells following 48h co-incubation in the presence of anti-TLR-2 blocking antibody. rH_{CC} in a moderate (10µg/ml) and high (20µg/ml) concentrations significantly induced IFN-γ production (P<0.001). The effect of rH_{CC} was also observed in the presence of anti-TLR-2 blocking antibody. However, rH_{CC} could not induce T cells to produce IFN-γ when it was used in low (2µg/ml) concentration (Figure 4).

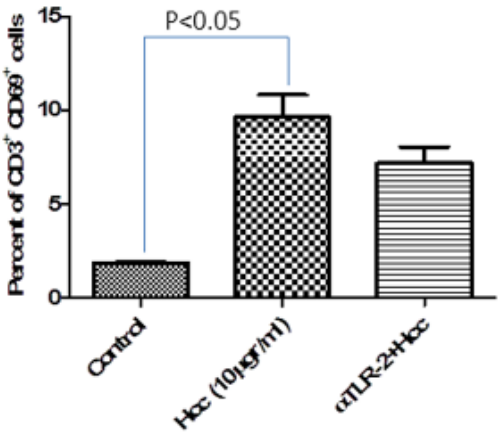

Figure 3. The overall percentages of CD3^{bright} and CD69^{bright} T cells co-incubated with 10 µgr/ml recombinant H$_{CC}$ in the presence or absence of anti TLR-2 inhibitory monoclonal antibody(p<0.05).

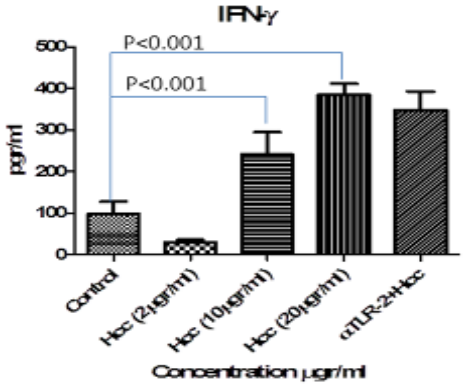

Figure 4. Enhanced IFN-γ production from T cells treated with recombinant H$_{CC}$ in presence of anti TLR-2 inhibitory antibody (p<0.001).

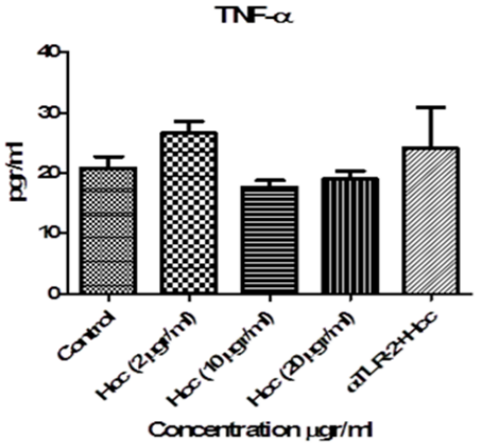

Figure 5. The capacity of recombinant H$_{CC}$ to stimulate TNF-α production from T cells in the presence or absence of anti TLR-2 inhibitory antibody.

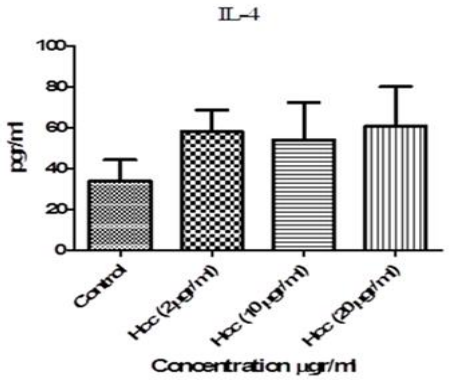

Figure 6. IL-4 secretion by T cells treated with rH$_{CC}$ for 24-48 hours.

rH$_{CC}$ Failed to Induce TNF-α Production in T Cells

The cytokine assessment revealed that rH$_{CC}$ was not able to induce TNF-α secretion form T cells even at high concentration (20µg/ml) (Figure 5).

Influence of Recombinant H$_{CC}$ on IL-4 Secretion Was Not Meaningful

No significant difference was observed in the IL-4 secretion from T cells treated with recombinant H$_{CC}$ in comparison with untreated cells (Figure 6).

DISCUSSION

In the present study, we investigated the ability of recombinant rH$_{CC}$ to induce T cells to express CD69 activation marker and produce IFN-γ, TNF-α and IL-4. We previously produced and characterized recombinant rH$_{CC}$.[13] For evaluation of the effects of rH$_{CC}$ on activation of T cells, we isolated and purified T cells from healthy individuals. Purified T cells were co-incubated with three different concentrations of rH$_{CC}$ in the presence or absence of anti-TLR-2 blocking antibody.

CD69 is the earliest activation market expressed on the surface of T lymphocytes followed by antigenic stimulation.[15] CD69 is able to fully block G-protein-coupled sphingosine 1-phosphate receptor-1 (S1P$_1$) which is needed for egression of lymphocytes from thymus and lymphoid organs.[16] Our results showed that recombinant H$_{CC}$ had a stimulatory effect on IFN-γ but not any impact on TNF-α and IL-4 secretions by T cells after 48h co-incubation in the presence of anti-TLR-2 blocking antibody. Our results indicated that

stimulatory effects of rH_{CC} on T cells were TLR-2 independent.

Many researchers showed that tetanus toxoid can induce clonal T cells activation.[14, 17, 18] Parronchi, et al.[10] demonstrated that TT specific T cells were mainly $CD4^+$ cells secreting Th1 cytokines such as IFN-γ. Adaptive immunity is essential for establishment of humoral immunity. Cytokines of Th1 CD4+ cells especially IFN-γ is the most potent mediator for isotype switching of B cells. Humoral immunity is the main immune mechanism providing total protection against tetanus. Active immunization is efficiently induced by tetanus toxoid and is crucial for the prevention of death caused by tetanus.[10] We previously showed that only the anti-fragment C antibodies dose-dependently can inhibit toxin binding to receptor.[19] Recently, we also demonstrated that most tetanus toxin specific monoclonal antibodies (seven out of eleven) were reactive to fragment C.[19] Fragment C can be considered as an immunodominant part of the toxin by which the humoral immune response is predominantly elicited. We also showed that six out of seven anti fragment C antibodies were able to inhibit TeNT binding to its receptor dose dependently.[19] According to our findings, rH_{CC} was critical for immunogenic activity of TeNT and could induce T cells in a TLR-2 independent manner. TLR-2 is widely expressed on the surface of T cells and is able to compose a heterodimer with TLR-1 and TLR-6. Among the toll like receptors, TLR-2 recognizes and binds to a variety of ligands including triacyllipopeptides, diacyllipopeptides, and peptides. In contrast to our study, many studies showed that microbial peptides activate T cells through TLR-2.[20-25]

T cell activation leads to cytokine secretion essential for isotype switching of B cells and production of IgG to protect host from tetanus. We suggest more investigation regarding the immunobiological properties of H_{CC} in tetanus.

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