Simultaneous determination of Human $CD4^+CD25^+$ regulatory T cells suppressing anti-TB immune responses of $CD4^+$, $CD8^+$ and $V\gamma2V\delta2^+$ T cells in vitro

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Abstract: To evaluate the negative regulatory effect of human $CD4^+CD25^+$ regulatory T cells (Treg) on anti-Tuberculosis (TB) immune responses of T cells including $CD4^+$, $CD8^+$ and $V\gamma2V\delta2^+$ T cells simultaneously in vitro, a versatile carboxyfluorescein succinimidyl ester (CFSE)-based proliferation assay was exploited to determine the suppressive effect of Tregs on the purified protein derivative (PPD)-specific proliferations of such T-subsets in peripheral blood mononuclear cells (PBMC) of BCG-infected donors. We demonstrated that PPD-stimulation droved synchronously the proliferations of both $CD4^+$ and $CD8^+$ T cells and $V\gamma2V\delta2^+$ T cells in PBMCs. Such PPD-specific proliferations of $CD4^+$, $CD8^+$ and $V\gamma2V\delta2^+$ T cells can be prohibited by human Treg concurrently. Similarly, Treg has the ability of suppressing the CD3/CD28 antibody-induced proliferations of such T-subsets. Our results indicated that the versatile CFSE-based proliferation assay can be applied to determine the complicated anti-TB immune response of multi-T-subsets simultaneously, and may facilitate human Tregs as potential tool to down-regulate overdue anti-TB immune responses involved in multiple effective T-subsets to enhance protection against *Mycobacterium tb* (*M. tb)* infection.

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1. Introduction

As one of the leading causes of death worldwide, tuberculosis (TB) is threatening human health seriously. There are more than 9 million new cases of TB and an estimated 2 million cases of deaths annually in the world. The most severe burden of TB disease are happening in developing countries(Lawn and Zumla, 2011). Notoriously, the shortage of the fundamental understanding of the pathogenesis and protective immunity against *Mycobacterium tb (M. tb)* and increasing rates of drug-resistant *M. tb* are the huge persisting challenges to public health worldwide, which greatly impede the promising progress in TB prevention and control (Lawn and Zumla, 2011). Therefore, it is urgently needed to seek and identify new reliable approaches to the prevention and treatment of TB based on exactly understanding the mechanisms of anti-TB immune responses.

The effectiveness of live Bacillus Calmette-Guerin (BCG) vaccination has been proved in against *M. tb* infection in children but not adult (Lawn and Zumla, 2011). As an attenuated strain of *M. bovis*, BCG encodes the same antigens of *M. tb* that can drive protective anti-*M. tb* immunity of human newborns via inducing potent specific and functional $CD4^+$ and $CD8^+$ T cell responses (Li et al., 2011). Therefore, elucidating BCG-elicited anti-TB immunity may facilitate us to find new effective measures to prevent and control *M. tb* infection(Gong et al., 2009; Qin et al., 2011).

T cells-mediated immunity plays a central role in host resistance to *M. tb* infection. It has been well documented over the past decades that the important role of $CD4^+$ T cells secreting cytokines such as IFN-γ, TNF-α and IL-2 to recruit monocytes, granulocytes and effective T cells migrating to disease sites and activate or amplify the effective functions of such immune cells to contributing collectively to antimicrobial immunity in *mycobacterial* infection (Li et al., 2011). Interestingly, the contributions of $CD8^+$ T cells(Chen et al., 2009) and Vγ2Vδ2 T cells(Chen, 2011; Shen et al., 2002) in *mycobacterial* infection have also been indicated recently. Likewise, growing studies revealed that these T cells contributed collaboratively to potent anti-*M. tb* immunity, for each of them may function as regulator to reciprocally enhance Ag-specific immune response of such T-subsets in infections (Chen, 2011).

Despite being a $CD4^+$ T cells, naturally occurring $CD4^+CD25^+$ regulatory T cells (Treg) play a key role in maintaining immune homeostasis in infection by suppressing the undue activation, proliferation and/or effective function of different types of T cells (Chen, 2011; Gong et al., 2009; Qin et al., 2011). Recently,

growing studies (Chen et al., 2012; Chen, 2011; Gong et al., 2009; Scott-Browne et al., 2007) had demonstrated that human Tregs have the negative regulatory effects on antigen-specific immune responses of independent $CD4^+$, $CD8^+$ T cells(Li et al., 2011) and $V\gamma2V\delta2^+$ T cells(Li and Wu, 2008) in *mycobacterial* infection. However, it is little known that Treg has the same effects on the anti-TB immune responses of a mixed pool of effective T cells including $CD4^+$, $CD8^+$ and $V\gamma2V\delta2^+$ T cells in peripheral blood mononuclear cells (PBMC) of *mycobacterial*-infected human, which reflects the delicate balance between such human T-subsets in clinical course and disease outcome in *M. tb* infections and the reality of human immune system in resisting pathogenic microorganism *in vivo*.

Accumulating evidence have indicated that Ag-specific immune response is very important in clearing various kinds of pathogens(Chen, 2011; Shen et al., 2002). To determine Ag-specific immune response during *M. tb* infection, numerous studies continued to rely on the ability of *M. tb*-specific T cells to produce cytokines, which may not accurately, completely and systematically reflect the functions of TB-specific T cells that including the activation, proliferation, division, autocrine and paracrine, etc. (Li et al., 2011). Attributable to further development in the application of flow cytometry, increased studies have showed that the multiple functions of Ag-specific poly-T-subsets can be assessed simultaneously by a versatile CFSE-based proliferation assay(Gong et al., 2009; Li et al., 2011; Qin et al., 2011). In the current study, we successfully conducted a CFSE-based proliferation assay to simultaneously determine the regulatory function of human Treg on anti-*M. tb* immune responses of $CD4^+$, $CD8^+$ and $V\gamma2V\delta2^+$ T cells in BCG-infected donors' PBMCs *in vitro*.

2. Material and Methods

2.1. Subjects

8 male healthy volunteers aged from 21 to 40 years old were enrolled from Zhengzhou University. 5~15 mL of EDTA-anticoagulant blood samples were collected from each volunteer. All protocols of this study were approved by the Biomedical Ethics Review Committee at Zhengzhou University, China.

2.2. BCG-infection

All volunteers were intradermally infected with BCG (Shanghai Institute of Biological Products, China) at a dose of 0.05mg in 0.1 ml volume within 3 months.

2.3. Cell isolation

PBMCs were separated from EDTA-anticoagulant peripheral blood using density gradient method. $CD4^+CD25^+$ T Cells were purified by using $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Miltenyi Biotec). Briefly, $CD4^+$ T cells were purified from PBMCs by depletion of non-$CD4^+$ cells with negative selection. From purified $CD4^+$ T Cells, $CD25^+$ T Cells were isolated by positive selection of $CD4^+CD25^+$ regulatory T cells using $CD25^+$ magnetic microbeads. $V\delta2^+$ T cells were purified by using purified mouse-anti-human Vδ2 (Clone: 15D, Endogen, Rockford, IL) Abs and goat-anti-mouse IgG microbeads (Miltenyi Biotec) with positive selection.

2.4. Labeling cells with CFSE and PKH26 red

Total 6×10^6 of $CD4^+CD25^+$ T cells-depleted PBMCs were labeled with CFSE using the CFSE Cell Proliferation Kit (Invitrogen-Molecular Probes) following the manufacturer's protocol. Briefly, the cells were suspended gently in 1 ml of prewarmed 0.1 % BSA -PBS containing CFSE at a 2.0 μM concentration and then incubated for 15 min at 37°C in dark. 5 volumes of ice-cold culture media were added to the cells and incubated 5 min on ice to quench the staining. Then the cells were washed by resuspending the pellet in fresh media for there times.

The purified 1×10^6 of $CD4^+CD25^+$ T cells were also labeled with PKH26 red using the PKH26 red fluorescent cell linker kit (Sigma) following the instructions. Briefly, the cells were suspended in a 2 ml total volume at final concentrations of 2×10^{-6} M PKH26 dye at room temperature for 5 minutes. Stop the staining reaction by adding an equal volume of complete medium. Then the cells were washed for total 4 times before being used.

2.5. CFSE-based proliferation assay

It was done following the standard protocols as previously described(Gong et al., 2009; Qin et al., 2011). The CFSE-labeled, $CD4^+CD25^+$ T cells-depleted PBMCs were added at 2×10^5 cells per well to individual wells of Costar round-bottom 96-well plates supplied with 0.2 ml of prewarmed R1640 containing with 10% FBS and 50 U/ml penicillin and 50 μg/ml streptomycin. Then these PBMCs were respectively stimulated by 15 μg/ml PPD (Shanghai Institute of Biological Products, China), or 5 ug/ml purified mouse anti-human CD28 (BD Pharmingen) and CD3 (BD Pharmingen). Meanwhile, PKH26 red labeled $CD4^+CD25^+$ T cells were then added to the CFSE-labeled, $CD4^+CD25^+$ T cells-depleted PBMCs at 2×10^5 per well in the absence or presence of PPD, CD28 and CD3.

After cultured for 7 days in the CO_2 incubator at 37℃, 95 % humidity and 5 % CO_2, the cells were collected and stained with surface Abs specific for Vγ2 (Endogen) in each tube at 4℃ for 20 min. After washed 3 times with 5% FBS-PBS, cells in each tube were added 5 uL Goat anti-mouse IgG (Biolegend) at 4℃ for 20 min in dark. Then wash 3 times again and add surface Abs specific for CD3 (Dako Cytomation), CD4 (BD Pharmingen), CD8 (Dako Cytomation) in each tube at 4℃ for 20 min in dark. Proliferation response was analyzed by flow cytometry to determine CFSE signal intensity and to exclude $PKH26^+$ cells, and the percentage of proliferation was calculated on the number of $CFSE^{dim}$

cells divided by the number of $CFSE^+$ cells. Flow cytometry was performed with CyAn ADP flow cytometer (DakoCytomation, Carpinteria, CA) and analyzed using Summit Data Acquisition and Analysis Software (DakoCytomation).

2.6. Statistical analysis

Unpaired student *t* test and one-way ANOVA were exploited to determine the differences between groups in vitro. p <0.05 was considered significant (GraphPad, San Diego, CA, USA).

3. Results

3.1. PPD induced antigen-specific proliferations of human $CD4^+$, $CD8^+$ and $V\gamma2V\delta2^+$ T cells

To determine whether human Treg has the function of suppressing the anti-*M. tb* immunity induced by effective T cells such as $CD4^+$, $CD8^+$ and $V\gamma2V\delta2^+$ T cells simultaneously, we used PPD as antigen-specific stimulation to induce the proliferations of $CD4^+$, $CD8^+$ and $V\gamma2V\delta2^+$ T cells in the PBMCs of volunteers that recently infected with BCG, since protein antigen of *M. tb* can be recognized directly by memory $CD4^+$ T cells, $CD8^+$ T cells and Vγ2Vδ2 T cells (Gong et al, 2009; Li et al, 2008; Qin et al., 2011).

We found that PPD induced the proliferations of not only $CD4^+$ T cells (p <0.01) and $CD8^+$ T cells (p <0.01), but also $V\gamma2V\delta2$ T cells (p <0.01) of BCG-infected volunteers (Figure 1, 2). Our results verify that human memory $V\gamma2V\delta2$ T cells directly recognized protein antigen of BCG, which is in accordance with the results in human study (Li et al, 2008).

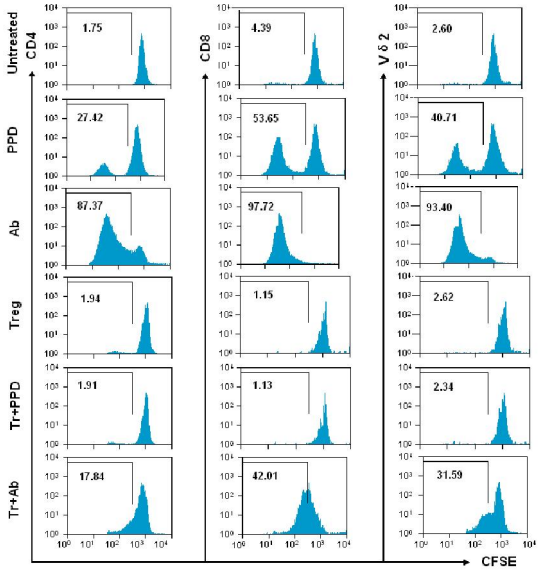

Figure 1. Hisgram figures of one representative volunteer of eight male volunteers showing PPD Ag-specific suppression of human $CD4^+CD25^+$ T cells on $CD4^+$, $CD8^+$ and $V\gamma2V\delta2^+$ T cells in PBMCs of BCG-infected donors in vitro. Tr: $CD4^+CD25^+$ T cells; Treg: $CD4^+CD25^+$ T cells.

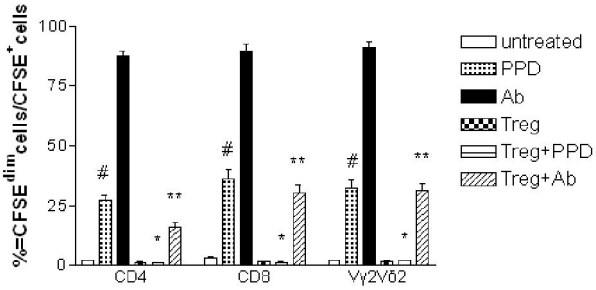

Figure 2. The suppressing percentage of $CD4^+CD25^+$ Treg on $CD4^+$, $CD8^+$ and $V\gamma2V\delta2^+$ T cells in vitro were shown as the mean values of triplicate measurements ± SEM (n=8): # Untreated *vs* PPD: $CD4^+$, $CD8^+$ and $V\gamma2V\delta2^+$ T cells, p <0.01 individually; * PPD *vs* Treg+PPD: $CD4^+$, $CD8^+$ and $V\gamma2V\delta2^+$ T cells, p <0.01 individually; ** Ab *vs* Treg+Ab: $CD4^+$, $CD8^+$ and $V\gamma2V\delta2^+$ T cells, p <0.001 individually.

3.2. Human Treg suppressed the PPD-specific proliferations of $CD4^+$, $CD8^+$ and $V\gamma2V\delta2^+$ T cells simultaneously

To further determine the suppression of human Treg on anti-*M. tb* immunity induced by effective T cells such as $CD4^+$, $CD8^+$ and $V\gamma2V\delta2^+$ T cells simultaneously, CFSE-based proliferation assay was exploited under the situation that Ag-specific immune responses of effective T cells can be driven by PPD-stimulation that results have been shown as above.

We found that Treg had the function of suppressing the PPD-specific induced activation/proliferation $CD4^+$ (p <0.01), $CD8^+$ (p <0.01) and $V\gamma2V\delta2^+$ T cells（p <0.01）, as well as the CD3/CD28 antibody-induced antigen non-specific proliferations of those T cells *in vitro* (p <0.001 individually) (Figure 1, 2).

4. Discussions

We conducted a sensitive, versatile CFSE-based proliferation assay for detecting the suppressing function of human Treg on anti-TB immunity *in vitro* (Gong et al, 2009; Li et al, 2008; Qin et al., 2011). A traditional assay for measuring such function of Treg is a coculture system by which a quantitive $CD4^+CD25^-$ responder T cells are incubated with different amounts of Treg under polyclonal stimulation. The proliferations of responder cells can be evaluated by measuring the incorporation of radioactive ^{3}H-thymidine into the DNA of cells in S phase during the last pulse 6~24h of culture. The advantages of CFSE-based method are obvious compared with ^{3}H-thymidine-based assay. Firstly, CFSE-based assay can be used directly to determine several phenotypes, surface or inner molecules such as cell cycle and cytokine profile of proliferated responder cells

simultaneously. At the same time, the proliferation of Treg droved by IL-2 in the coculture is excluded and then the suppressive mechanisms of that can be further analyzed. Secondly, CFSE-based assay is more sensitive than ^{3}H-thymidine-based assay which have high levels of background for the detection of rare antigen-specific T cell responses(Venken et al., 2007). The proliferation detected by later method is often underestimated. Thirdly, the proliferation of CFSE-based assay can be analyzed directly by flow cytometry to avoid using radioactive materials and machines. Fourthly, ^{3}H-thymidine-based assays can not distinguish proliferations of different cell populations from mixed pool, but CFSE-based method can.

For the past decades, the evaluations on the suppressing function of Treg focused on its inhibiting role in the proliferation of a single cell types, for example, $CD4^{+}CD25^{-}$ effective T cells. Thanks to the recent rapid progress in the regulated targets of Treg, effective $CD8^{+}$ and $V\gamma2V\delta2^{+}$ T cells were added into the pool of its regulatory objects that covered almost all the current types of immune cells. And that the cooperative and competitive roles of these T-subsets in immunity against *M. tb* were also indicated in the recent studies(Chen et al., 2009; Gong et al., 2009; Li and Wu, 2008). Therefore, we decided to determine the *M. tb* Ag-specific and non-specific proliferations of $CD4^{+}$, $CD8^{+}$ and $V\gamma2V\delta2^{+}$ T cells in the PBMCs of BCG-infected donors with CFSE-based proliferation assay. Indeed, our results were in agreement with references that $CD4^{+}$, $CD8^{+}$ and $V\gamma2V\delta2^{+}$ T cells contributed simultaneously to anti- *M. tb* immunity because the PPD-specific proliferations of such T-subsets can be observed *in vitro*. In addition, our results supported that CFSE-based proliferation assay can be applied to detect concurrently the Ag-specific immune responses of multiple effective T-subsets, which will make it easy to probe deeply into the mechanisms of extensive anti-mycobacterium immunity mediated by poly-T-subsets *in vitro*.

Furthermore, by flow cytometric analysis of CFSE-based proliferation assay, we found the powerful suppressing functions of Treg on $V\gamma2V\delta2$ T cells, as well as $CD4^{+}$, $CD8^{+}$ T cells in BCG-infected human. Treg did suppress not only the PPD-specific proliferation of $CD4^{+}$ T cells which play most important roles in anti-*M. tb* immunity, but also that of $CD8^{+}$ and $V\gamma2V\delta2^{+}$ T cells. Likewise, Tregs suppressed not only PPD Ag-specific, but also CD3/CD28 antibody-induced non-specific proliferations of conventional $CD4^{+}$, $CD8^{+}$ T cells, and nonclassical $V\gamma2V\delta2$ T cells, which suggested Treg may control anti-mycobacterium immunity that mediated not only by conventional $CD4^{+}$, $CD8^{+}$ T cells, but also by nonclassical $V\gamma2V\delta2$ T cells during anti- *M. tb* immunity. These evidences demonstrated that human Treg has versatile regulatory functions on a mixed pool of effective cells including $CD4^{+}$, $CD8^{+}$ T cells (Chen et al., 2009; Chen et al., 2012) and $V\gamma2V\delta2$ T cells (Chen et al., 2012; Gong et al., 2009; Li and Wu, 2008) that contribute collectively to the extensive ongoing anti-mycobacterium immunity.

The results of this study may open up the possibilities to exploit Treg as potential tool for immunotherapy during infectious or other obstinate diseases. For example, pre-activated Treg may be considered to be used for treating acute TB with brain and pulmonary lesion caused by overactive effective memory T cells which contribute to excessive immune response to result in cerebrocortical necrosis and pulmonary cavity(Chen et al., 2012).

In summary, our results demonstrated that human Treg had the function of suppressing the anti-*M. tb* immunity of multiple effective T cells such as $CD4^{+}$, $CD8^{+}$ and $V\gamma2V\delta2^{+}$ T cells that detected simultaneously by the versatile CFSE-based proliferation assay (Chen, 2011; Li and Wu, 2008; Scott-Browne et al., 2007; Shen et al., 2002; Qin et al., 2011). The results may facilitate the clinical application of Treg in controlling and treating intractable *M. tb* infections, and the further application of CFSE-based proliferation assay to detect Ag-specific immune responses of multiple cells during infectious diseases.

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