$CD4^+CD25^+$ regulatory T cells suppress the *M.TB*-specific immune response of T cells

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Abstract: To investigate the negative regulatory effect of macaques $CD4^+CD25^+$ regulatory T cells (Tregs) on anti-*M.TB* immune response of memory T cells in vitro through determining the suppression of Tregs on the purified protein derivative (PPD)-specific proliferation of memory T cells. Peripheral bloods were drawn from 6 male *Rhesus macaques (RH)* adminimistrated by BCG within 3 months, and then $CD4^+CD25^+$ T cells were separated from PBMCs by immunomagnetic beads and labeled with PKH26 red. Cultured the remain $CD4^+CD25^+$ T cells-depleted, CFSE-labeled PBMCs alone or in the presence of purified $CD4^+CD25^+$ T cells to 8th day and then both were stimulated by PPD antigen or purified CD3, CD28 antibodies respectively. The proliferations of $CD4^+$ T cells, $CD8^+$ T cells and Vγ2Vδ2 T cells in PBMCs were analyzed by flow cytometry to determine the dilution of CFSE fluorescence intensity and to exclude $PKH26^+$ cells: the percentage of proliferation was calculated on the number of $CFSE^{dim}$ cells divided by the number of $CFSE^+$ cells. The results showed that PPD droved the proliferations of not only memory $CD4^+$ T cells (p <0.001) and $CD8^+$ T cells (p <0.01), but also Vγ2Vδ2 T cells (p <0.05). Besides suppressing the CD3/CD28 antibody-induced non-specific proliferations of $CD4^+$ T cells (p <0.001), $CD8^+$ T cells (p <0.01) and Vγ2Vδ2 T cells (p <0.05) significantly, $CD4^+CD25^+$ T cells have the function of suppressing the PPD-specific proliferations of $CD4^+$ T cells (p<0.001) and $CD8^+$ T cells (p <0.01), Vγ2Vδ2 T cells (p <0.05). These results suggested that Tregs of macaques have negative regulatory effect on anti-*M.TB* immune response of memory T cells in vitro.

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1. Introduction

Among infectious diseases, tuberculosis is still one of the major fatal diseases in the world (Huang et al, 2007): there are 8～9 million new cases of TB and 1.5～2.5 million cases of deaths annually in the world (Li et al, 2007). Attenuated BCG has been proved effective in preventing children tuberculosis in developing countries (Soysal et al, 2005). However, it is not very clear of the mechanisms about pathogenesis and anti-*M.TB* immunity, which greatly impedes the improvement of TB prevention and control. Therefore, that elucidating anti-*M.TB* immunity triggered by BCG infection will be very beneficial to find new effective measures to prevent and control *mycobacterium tuberculosis* infection (Huang et al, 2007).

Tregs play key role in the process of maintaining immune balance during infection, tumor and autoimmune diseases by suppressing the activation, proliferation and/or effective function of effective T cells (Li et al, 2007). Our (Gong et al, 2009) and other's studies (Li et al, 2007/2008; Chen et al, 2007; Scott-Browne et al, 2007) recently indicated that Tregs may have the negative regulating function in primate anti-*M.TB* immunity.

Because their biological characteristic are very close to human, non-human primate have been used as precious human infectious disease model. Of most concern, studying the anti-infectious immunity of non-human primates with human infectious disease will be of great significance and importance in reality in preventing and controlling human mycobacterium tuberculosis. In this study, we determined the regulatory function of non-human primate Tregs on effective T cells such as $CD4^+$ T, $CD8^+$ T cells and Vγ2Vδ2 T cells during anti-M.TB immunity *in vitro*.

2. Material and Methods

Animals

6 male RH macaques were provided by Biological Resource Center in the University of Illinois at Chicago (UIC). At beginning of the study, their ages ranged from 3 to 10 years old and body weight from 2.3 to 15.1 kg. All animals were maintained and used in accordance with guidelines of Institutional Animal Care and Use Committee (IACUS) at UIC. Animals were anesthetized with 10 mg/kg ketamine HCl (Fort Dodge Animal Health, Fort Dodge, IA) i.m. before drawing blood samples and being treated. All monkeys were artificially introvenous infected with BCG at a

dose of 10^7 in 0.5 ml volume within 3 months.

Cell isolation

5~ 15 mL of EDTA blood samples were collected from each macaque before and after BCG infection. Then 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 for non-human primate (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.

Label Cells with CFSE and PKH26

6×10^6 of $CD4^+CD25^+$ T cells-depleted PBMCs were prepared and 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 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. The cells were then washed for total 4 times before being used.

CFSE-based proliferation Assay

For the suppression assay, the CFSE-labeled, $CD4^+CD25^+$ T cells-depleted PBMCs were added at 2×10^5 cells/well to individual wells of Costar round-bottom 96-well plates supplied with 0.2 ml of prewarmed R1640 with 10% FBS, 50 U/ml penicillin and 50 μg/ml streptomycin and stimulated by 15 μg/ml PPD (Colorado Serum Company), or 5 ug/ml purified mouse anti-human CD28 (CD28.2, BD Pharmingen) and CD3 (SP34.2, BD Pharmingen). Meanwhile, PKH26 labeled $CD4^+CD25^+$ T cells were then added to the CFSE-labeled, $CD4^+CD25^+$ T cells-depleted PBMCs at 2×10^5 /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 then stained with surface Abs specific for Vγ2 (7A5, 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-APC (Biolegend) at 4℃ for 20 min in dark. After washed 3 times, add surface Abs specific for CD4-Pacific Blue (OKT-4, eBioscience), CD8-PECy7 (DK25, DakoCytomation) 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: the percentage of proliferation was calculated on the number of $CFSE^{dim}$ cells divided by the number of $CFSE^+$ cells.

Statistical analysis

Student *t* test was exploited to determine the differences between groups in vitro. $p<0.05$ was considered significant (GraphPad, San Diego, CA, USA).

3. Results

PPD induced antigen-specific proliferation of memory $CD4^+$ T cells, $CD8^+$ T cells and Vγ2Vδ2 T cells

To determine whether Tregs had the function of suppressing the anti-*M.TB* immunity induced by effective T cells such as $CD4^+$ T cells, $CD8^+$ T cells and Vγ2Vδ2 T cells, we use PPD as antigen-specific stimulation to induce the proliferation of $CD4^+$ T cells, $CD8^+$ T cells and Vγ2Vδ2 T cells in the PBLs of *RH macaques* that recently infected with BCG since protein antigen can be recognized directly by memory $CD4^+$ T cells, $CD8^+$ T cells and Vγ2Vδ2 T cells (Gong et al, 2009; Li et al, 2008).

We found that PPD induced the proliferation of not only $CD4^+$ T cells ($p<0.001$) and $CD8^+$ T cells ($p<0.01$), but also Vγ2Vδ2 T cells ($p<0.05$) of BCG-infected *macaques* (Figure 1, 2), excepting BCG-naive *macaques* (data not show). Our results verify that *macaque* memory Vγ2Vδ2 T cells recognized directly protein antigen, which is in accordance with results in human study (Li et al, 2008).

Tregs suppressed the PPD-specific proliferation of memory $CD4^+$ T cells, $CD8^+$ T cells and Vγ2Vδ2 T cells

To further determine whether Tregs had the function of suppressing the anti-*M.TB* immunity induced by effective T cells such as $CD4^+$ T cells, $CD8^+$ T cells and Vγ2Vδ2 T cells, CFSE-based proliferation assay was exploited under the situation that anti-*M.TB* immunity of effective T cells can be

driven by PPD that results have been shown as above.

We found that Tregs have the function of suppressing the PPD-specific induced activation/proliferation of $CD4^+$ T cells, $CD8^+$ T cells and Vγ2Vδ2 T cells（p<0.001~0.05）, as well as the CD3/CD28 antibody-induced antigen non-specific proliferation of those cells *in vitro* (p<0.001 individually) (Figure 1, 2).

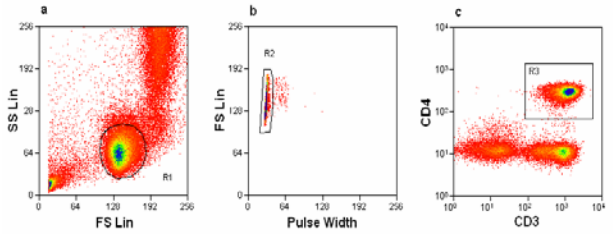

Figure 1. Taking $CD3^+CD4^+$ T cells for example to show the gating strategy. Lymphocytes were gated based on forward and side scatters, and pulse width, and at least 50 000 gated events were analyzed (a→b); Further special gates and quadrants were determined based on nonstaining, isotype control Ab for background staining and specific Ab staining (c). R3 area in figure c represents $CD3^+CD4^+$ T cells gated from R2 area in figure b that is gated from R1 area in figure a.

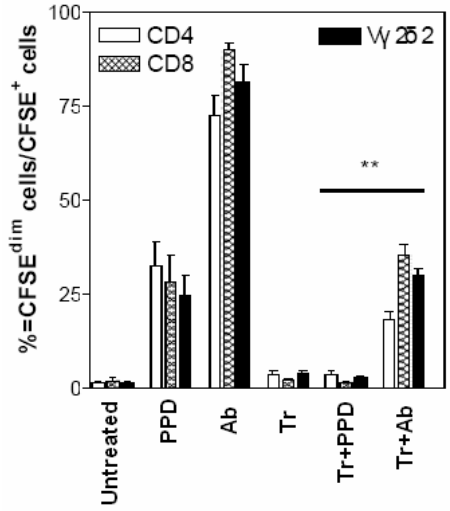

Figure 2. The percentage of suppression of $CD4^+CD25^+$ Tregs on $CD4^+$ T cells, $CD8^+$ T cells and Vγ2Vδ2 T cells in vitro are shown as the mean values of triplicate measurements ±SEM (n=6): ** PPD vs Tr+PPD: CD4, p<0.001; CD8, p<0.01; Vγ2Vδ2, p<0.05. Ab vs Tr+Ab: CD4, CD8 and Vγ2Vδ2, p <0.001.

4. Discussion

In the present study, we conducted a sensitive, versatile CFSE-based assay for detecting the suppressing function of *macaque* Tregs *in vitro* (Gong et al, 2009; Li et al, 2008). By flow cytometric analysis of CFSE-based assay, we found the powerful suppressing functions of Tregs on Vγ2Vδ2 T cells, as well as $CD4^+$, $CD8^+$ T cells in BCG infected *macaque*. Tregs 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 $CD8^+$ T cells and Vγ2Vδ2 T cells. Furthermore, Tregs suppressed not only PPD antigen-specific, but also CD3/CD28 antibody-induced non-specific proliferation of conventional $CD4^+$, $CD8^+$ T cells, and Vγ2Vδ2 T cells, which suggested Tregs may control anti-*mycobacterium* immunity that mediated not only by conventional $CD4^+$, $CD8^+$ T cells, but also by Vγ2Vδ2 T cells during anti-*M.TB* immunity. These evidences demonstrated that *macaque* Tregs have versatile regulatory functions on a pool of effector cells including $CD4^+$, $CD8^+$ T cells (Chen et al, 2009) and Vγ2Vδ2 T cells (Gong et al, 2009; Li et al, 2008) which contribute collectively to the extensive ongoing anti-mycobacterium immunity.

Our results provide the first-hand evidence about *macaque* Tregs controlling anti-*M.TB* immunity induced by effective memory T cells, which will further promote the application of *macaques* in the study of human mycobacterium-infected diseases. What's more, the results of this study open up the possibilities to exploit Tregs as potential tool for immunotherapy during infectious or other diseases. For example, pre-activated Tregs may be considered to be used in the future treating acute tuberculosis 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.

In conclusion, our results demonstrated that *macaque* Tregs had the function of suppressing the anti-*M.TB* immunity induced by effective T cells such as $CD4^+$ T cells, $CD8^+$ T cells and Vγ2Vδ2 T cells which is assistant with other studies (Shen et al, 2002; Barboza et al, 2007; Boettler et al, 2005; Cavassani et al, 2006), and will further promote the clinical application of Tregs in controlling and treating intractable infectious diseases.

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