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CD8$^+$ T activation attenuates CD4$^+$ T proliferation through dendritic cells modification

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ABSTRACT

Emerging evidence has suggested that CD8$^+$ T had modulatory function on CD4$^+$ T mediated autoimmune and inflammatory diseases. However, the underlying mechanisms remain unclear. In this study, we found that CD8$^+$ T activation inhibited $OVA_{323-339}$ antigen specific CD4$^+$ T cells proliferation *in vitro* and *in vivo*. Further investigation demonstrated that this immunosuppression largely depended on the soluble factor from activated CD8$^+$ T to modify the phenotype and functions of DCs. Moreover, not only the inhibitors for IDO or iNOS, but also IFN-γ neutralization markedly reversed this immunosuppression on $OVA_{323-339}$ antigen specific CD4$^+$ T cells proliferation. Interestingly, CD8$^+$ T cells absence aggravated the pathological damage in lung in OVA-induced asthma model, but alleviated by CD8$^+$ T transfer and activation. Thus, these findings suggested that activated CD8$^+$ T population exerted feedback regulation in DCs modification, and then attenuated CD4$^+$ T mediated immune response.

1. Introduction

As one system with complicated biological structures and processes, the immune system relies on sophisticated regulatory networks to maintain homeostasis. In recent decades, a variety of immunoregulatory factors have been widely reported, especially the immunomodulatory functions of regulatory CD4$^+$ T cells. Comparatively, the counterparts of CD8$^+$ T cells are less defined but attracting increasing attention.

CD8$^+$ T cells are generally recognized as a cytotoxic T-cell population, which plays a critical role in anti-viral infection, tumor cell elimination, and allograft rejection. In contrast, accumulating CD8$^+$ T subsets with immunoregulatory/immunosuppressive capacities have been reported in CD4$^+$ T-associated autoimmune and inflammatory diseases [1], including asthma [2], systemic lupus erythematosus (SLE) [3], inflammatory bowel disease (IBD) [4,5], and experimental autoimmune encephalomyelitis (EAE) [6,7]. Several potential mechanisms for the immunoregulation of these CD8$^+$ T on CD4$^+$ T-mediated immunity have been explored, such as immune regulatory cytokines secretion (e.g., IL-10, TGF-β, IFN-γ) [8], cell–cell direct contact [9,10], and cytotoxic lysis of APCs in a perforin-dependent or Fas-dependent manner [11,12]. Collectively, these data suggested that CD8$^+$ T could exert regulatory functions in immunity through multiple mechanisms.

As a type of professional antigen presenting cells, dendritic cells (DCs) play pivotal roles in CD4$^+$ T activation and proliferation. The phenotypes and functions of these cells are highly plastic and modifiable by factors in the ambient environment [13,14]. Our and other's studies have shown that some special microenvironments can drive mature and immature DCs to differentiate into regulatory DCs, thereby suppressing their ability to induce CD4$^+$ T responses [15–18]. Activated CD8$^+$ T cells express many adhesion molecules on the cell membrane, and secret different cytokine profiles from its quiescent state. These adhesion molecules and cytokines work as indispensable mediators involving in the cross-talk of immune cells in immune response. Therefore, in a similar fashion, activated CD8$^+$ T cells might generate a special immune microenvironment, and indicated their potential regulatory functions on DCs, and then

Abbreviations: 1-MT, 1-methyl-tryptophan; BAL, bronchoalveolar lavage; CFSE, 5(6)-carboxyfluorescein diacetate N-succinimidyl ester; CMFDA, 5-chloromethylfluorescein diacetate; DC, dendritic cell; Far-Red, CELLTRACE(TM) FAR RED DDAO-SE; HE, hematoxylin and eosin; IDO, indoleamine-pyrrole 2,3-dioxygenase; IFN-γ, interferon-γ; IL, interleukin; iNOS, inducible nitric oxide synthase; OVA_{pep}-loaded DCs, $OVA_{323-339}$ + $OVA_{257-264}$-loaded DCs; PAS, periodic acid-Schiff; PBIT, S,S′-(1,3-phenylenebis[1,2-ethanediyl]) bisisothiourea; pSTAT3, phosphorylated signal transducer and activator of transcription 3; SN, supernatant isolated from the activated CD8$^+$ T cells; SN-modified DCs, supernatant-modified DCs; TGF-β, transforming growth factor-β.

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ultimately on the activation and proliferation of $CD4^+$ T cells. But, up to now, the exact roles of activated $CD8^+$ T in immune regulation are not well demonstrated.

In this study, we explored the functions of activated $CD8^+$ T in immune regulation by using T-cell/DC co-culture *in vitro* and $CD8^+$ T adoptive transfer and activation *in vivo*. Indeed, we found that $CD8^+$ T activation negatively regulated $CD4^+$ T cells proliferation through altering the phenotypes and functions of DCs. Thus, our findings provided a new clue to clarify the crosstalk between T cells and DCs, and indicated the potential regulatory roles of activated $CD8^+$ T in allergic airway inflammation.

2. Materials and methods

2.1. Ethics statement

All experimental protocols were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of Tsinghua University, Beijing, China. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize animal suffering.

2.2. Mice

All mice were bred and maintained in specific, pathogen-free conditions at the experimental animal platform of Tsinghua University, and were used at 6–8 weeks of age. C57BL/6 and BALB/c mice were obtained from Chinese Academy of Medical Sciences (CAMS). OT-1 transgenic (Tg) mice [19], and OT-2 Tg mice [20] were provided by Charles River Laboratories (Wilmington, MA, USA). β2m null mice, which are deficient in MHC class I protein expression and have few $CD8^+$ T cells [21]; DO11.10 mice (BALB/c background) carrying Tg CD4 TCR specific for H_2K^d-$OVA_{323–339}$ residues [20]; IFN-γR(−/−) mice on C57BL/6 genetic background [22]; and C.Cg-$Foxp3^{tm2Tch}$/J mice on BALB/c genetic background (in which 97% of $Foxp3^+$ T cells are identifiable by $EGFP^+$) were all purchased from the Jackson Laboratory (Bar Harbor, ME, USA).

2.3. Reagents

All cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin. RPMI 1640 medium and fetal calf serum (FCS) were purchased from PAA Laboratories (Cölbe, Germany). Recombinant murine cytokines GM-CSF, IL-4, IL-6, IFN-γ were purchased from ProsPec (Otisville, NY, USA). Fluorescein-conjugated monoclonal antibodies and isotype control antibodies were purchased from Biolegend (San Diego, CA, USA). Neutralizing antibodies to IFN-γ and TGF-β were purchased from R&D Systems (Minneapolis, MN, USA). Anti-CD4, CD8 and CD11c magnetic microbeads were provided by Miltenyi Biotec (Bergisch Gladbach, Germany). Primary antibodies to iNOS and pSTAT3 were purchased from Cell Signaling Technology (Beverly, MA, USA), anti-IL-10 and anti-TGF-β were from R&D Systems, IDO antibody was purchased from Biolegend, and anti-IL-12p40 was provided by eBioscience (San Diego, CA, USA). CFSE, CMFDA, Far-Red and propidium iodide (PI) were obtained from Invitrogen (Carlsbad, CA, USA). Ovalbumin (OVA, grade V), $OVA_{257–264}$ (OT-1 peptide), $OVA_{323–339}$, PBIT and 1-MT were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.4. Dendritic cells (DCs) preparation

Bone Marrow derived mature DCs (maDCs) were prepared from C57BL/6, BALB/c or IFN-γR(-/-) mouse bone marrow according to the method described previously [15]. To prepare OVA loaded DCs, maDCs were incubated with 400 ng/mL $OVA_{257–264}$ and/or 1 μg/mL $OVA_{323–339}$ peptides for 24 h. After residual free peptides were removed, peptide-loaded DCs were resuspended in PBS and then were infused i.v. at a dose of 1.5×10^6 per mouse 24 h after T cells being transferred. To prepare supernatant modified DCs, OT-1 $CD8^+$ T (1×10^6) co-cultured with maDCs (1×10^5) for 36 or 60 h, and supernatant (SN) was collected. Supernatant modified DCs (SN-modified DCs) were prepared by incubating OVA loaded maDCs with activated $CD8^+$ T derived supernatant and fresh RPMI-1640 complete medium (1:1, v:v) for 48 h. In some experiments, $CD11c^+$ splenic DC cells were used.

2.5. Flow cytometry analysis

The phenotype of immune cells, phagocytic ability of maDCs and T cells proliferation were assayed by using flow cytometry (FCM) (FACSAria II, B.D. Biosciences) as described previously [15]. The data were analyzed using FlowJo software (TreeStar Inc.; Ashland, OR, USA).

2.6. The purification and proliferation assays of T cells

$CD4^+$ and $CD8^+$ T cells were purified by positive selection with magnetic-activated cell sorting (MACS). The purity of cells was routinely more than 90%.

For $CD4^+$ T proliferation assay *in vitro*, 2×10^5 CFSE-labeled OT-2 $CD4^+$ T cells were co-cultured with 2×10^4 OVA_{pep}-loaded maDCs in the presence or absence of 1×10^5 OT-1 $CD8^+$ T cells in triplicate in 96-well round-bottom plates. The divisions of $CD4^+$ T were assessed 72 or 96 h later by using FCM analysis. In some experiments, IDO inhibitor 1-MT (200 μM), iNOS inhibitor PBIT (10 μM) or TGFβ neutralizing antibodies (10 μg/mL) were added. For $CD4^+$ T proliferation assay *in vivo*, 4×10^6 CFSE-labeled OT-2 $CD4^+$ T cells were transferred i.v. with or without 2×10^6 OT-1 $CD8^+$ T cells into C57BL/6 recipient 24 h prior to 1.5×10^6 peptide-loaded maDCs challenge. Four days later, the percentage of $CFSE^+CD4^+$ T cells in total live $CD4^+$ T in the spleen and blood was assessed by FCM. In some experiments, $CD4^+$ T cells from DO11.10 × C57BL/6 F1 hybrid mice were transferred i.v. into BALB/c × C57BL/6 F1 hybrid mice 24 h prior to peptide-loaded DCs injection, which were treated by activated $CD8^+$ T cells supernatant. Four days after DCs challenge, the proliferation of $CD4^+$ T was assessed by determining the percentages of $KJ1\text{-}26^+CD4^+$ T cells in total live $CD4^+$ T cells in the spleen and blood sample in recipient using FCM.

2.7. $Foxp3^+$ Treg differentiation assay in vitro

$CD4^+$ T-cell and $CD8^+$ T-cell were isolated from DO11.10 × $Foxp3^{EGFP}$ F1 hybrid mice and OT-1 × Balb/C F1 hybrid mice, respectively. $CD4^+$ T cells were co-cultured in the presence or absence of $CD8^+$ T-cell with OVA_{pep}-loaded maDCs for 48 h, and then were collected to detect the frequency of $CD4^+CD25^+Foxp3^+$ T cells (Treg cells) among total $CD4^+$ T cells.

2.8. Cytokines profile analysis

OT-1 $CD8^+$ T (2×10^6) and $OVA_{257–264}$-loaded maDCs (2×10^5) were co-cultured for 36 h or 60 h in 24-well plates (2 mL per well). Supernatants were collected, and frozen at −80 °C for further assay. The levels of cytokines in supernatants were analyzed using a mouse Th1/Th2/Th17/Th22 13plex flowcytomix assay kit (eBioscience; San Diego, CA, USA) on the FACSAria II (BD Biosciences; San Jose, CA, USA). Flowcytomix™ Pro Software was used for data acquisition and analysis.

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