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Short communication

MHC class II peptides induce $CD8^+CD44^+Ly49^+$ regulatory T cells in C57BL/6 mice

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ABSTRACT

$CD8^+$ regulatory T cells (Tregs) play an important role in regulating peripheral immune tolerance. However, difficulties in the characterization of $CD8^+$ Tregs that lack suitable markers have a considerably limited research in this area. Moreover, the induction and effector mechanisms of $CD8^+$ Tregs remain unclear. Herein, we demonstrate the suitability of Ly49 and CD44 as markers for $CD8^+$ Tregs. Our data also show that MHC class II restricted peptides induce $CD8^+CD44^+Ly49^+$ Tregs via $CD4^+$ T cell activation. Furthermore, we also found cross-suppressive activity of these $CD8^+$ Tregs on responding $CD4^+$ T cells in a cytotoxicity dependent manner. Our data provide new insights into the induction and function of $CD8^+$ Tregs.

1. Introduction

The adaptive immune system maintains a balance between the induction of protective immunity against pathogens and self-tolerance. Although negative selection deletes strongly self-reactive T cells [1], this process is incomplete. A significant fraction of T cells that express receptors with intermediate or low affinity for self-antigens are spared, and these can differentiate into effector cells in the context of inflammatory stimulation [2–4]. In addition to negative selection, self-tolerance also depends upon inhibitory interactions between effector T cells and regulatory or suppressive cells in the periphery [5]. This process is held in check by several cellular mechanisms, including $CD4^+CD25^+FoxP3^+$T cells, which are specialized regulatory T cells (Tregs) [6]. Furthermore, a sub-lineage of $CD8^+$ T cells was initially shown to display immuno-regulatory activity on activated $CD4^+$ T cells, which is termed $CD8^+$ T regulation [7].

We have previously demonstrated that glatiramer acetate – an immunomodulator approved for the treatment of multiple sclerosis (MS) – ameliorates inflammatory bowel disease (IBD) in murine models through the induction of a subset of Qa-1 restricted $CD8^+$ Tregs [8]. However, owing to the paucity of reliable markers, we were unable to distinguish $CD8^+$ Tregs from conventional $CD8^+$ T cells. Since then, several studies have noted that T cell memory markers CD44 and Ly49 – an MHC class I-specific inhibitory receptor in mice – are specifically expressed on $CD8^+$ T regulatory [9–12].

This finding suggested that Ly49 may be an ideal marker for this subset of $CD8^+$ T cells. Furthermore, although some studies had indicated that $CD8^+$ Tregs were up-regulated by activated $CD4^+$ T cells [13–15], it is still uncertain whether this subset of cells was induced by only the auto-reactive target $CD4^+$ T cells or all activated $CD4^+$ T cells.

In the current study, we employed a peptide-immunized model to further illustrate the induction and effector mechanisms of $CD8^+$ Tregs, and found an increased proportion of $CD8^+CD44^+Ly49^+$ Tregs in mice immunized with MHC class II restricted peptides – regardless of the amino acid sequence and species origin of these peptides. Furthermore, these $CD8^+CD44^+Ly49^+$ Tregs showed cross-suppressive activity of OT2 $CD4^+$ T cells, which were specifically activated by $OVA_{323\text{-}339}$, and their suppressive activity depended on its cytotoxicity mediated by perforin. Our work has shown that $CD8^+CD44^+Ly49^+$ Tregs could be induced by activated $CD4^+$ T cells, revealing a new insight into the induction and function of $CD8^+$ T regulatory cell.

Abbreviations: Treg, T regulatory cells; n.s, not significant; CFA, complete Freund's adjuvant; RAA, random amino acid sequences.

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2. Materials and method

2.1. Animals

Female mice of 8–10 weeks of age were used. C57BL/6 mice were purchased from Shanghai SLAC Laboratory Animals Co., Ltd (Shanghai, China). *H2*-deficient (B6.129S2-*H2*$^{dlAb1\text{-}Ea}$/J), *perforin 1*-deficient (C57BL/6-*Prf1*^{tm1Sdz}/J), *FasL*-deficient (B6Smn.C3-*Fasl*^{gld}/J) and OT-2 (B6.Cg-Tg(TcraTcrb)425Cbn/J) mice were from Jackson Laboratory (Bar Harbor, ME, US), that were crossed on the C57BL/6 background. All mice were co-housed for >4 weeks in the pathogen-free facility at Zhejiang University Laboratory Animal Center. Experimental protocols were approved by the review committee of the School of Medicine at Huzhou University, and were conducted in compliance with the institutional guidelines.

2.2. Peptides

Three MHC class I-restricted (H-2K^b), three MHC class II-restricted (I-A^b) and random amino acid (RAA) sequence peptides were synthesized by Sangon Corp (Shanghai, China) [16–21]. All peptides were >98% pure, according to RP-HPLC analysis. The $OVA_{323\text{-}339}$ peptide (ISQAVHAAHAEINEAGR) was from Invivo-Gen (San Diego, CA, USA). A summary of all peptides is presented in Table 1.

2.3. Immunization

Peptides (2 mg/mL) were individually emulsified with an equal volume of complete Freund's adjuvant (CFA, Sigma-Aldrich, St. Louis, USA) at a final concentration of 1 mg/mL. Next, 200 μL of emulsified peptides was injected into two footpads of each C57BL/6 mouse and 14 days after the first immunization, the mice were re-stimulated with the same dose of peptides. Twenty-eight days after the first immunization, the spleen and peripheral lymph node cells were harvested from the immunized mice.

2.4. Antibodies and reagents

FITC-conjugated anti-mouse CD8α (53–6.7), PE-conjugated anti-mouse CD3 (145–2C11), PE-conjugated anti-mouse Ly49C/F/I/H (14B11), PE-conjugated anti-mouse Vβ5.1, 5.2 (MR9-4), PE/Cy5-conjugated anti-mouse/human CD44 (IM7), PE/Cy5-conjugated anti-mouse CD4 (GK1.5), PE/Cy7-conjugated anti-mouse CD25(PC61), APC-conjugated anti-mouse CD122 (TM-β1), APC-conjugated anti-mouse CD3 (145–2C11), APC-conjugated anti-mouse CD8α (53–6.7) and Alexa Fluor 647-conjugated anti-mouse/rat/human Foxp3 (150D) antibodies were from BioLegend (San Diego, CA, USA). Mouse IFN-γ ELISA Ready-SET-Go!® was from eBioscience (San Diego, CA, USA).

2.5. Flow cytometry

Single-cell suspensions were prepared from the spleen and peripheral lymph nodes and maintained in the dark at 4 °C for subsequent immuno-fluorescence analysis. Cells were washed in ice-cold FACS buffer (2% FCS and 0.1% NaN3 in PBS), incubated with each antibody for 30 min, and then washed in FACS buffer before analysis.

For proliferation analysis, target T cells were gated on CD4$^+$CFSE$^+$. The percentage of cells that divided at least once (% divided, top left corner) and the average number of cell divisions (division index, bottom left corner) are indicated in the figure corresponding to each sample.

The True-Nuclear™ Transcription Factor Buffer Set (San Diego, CA, USA) was used for intracellular staining of the transcription factor FoxP3. All operations were analyzed using a BD FACSCalibur™ analyzer (BD Biosciences) and FlowJo software (TreeStar, Ashland, OR, USA).

2.6. Cell enrichment

T lymphocytes were harvested from the spleen and peripheral lymph nodes (PLNs) of C57BL/6 mice immunized with the peptide mixture and enriched with T Lymphocyte Enrichment Set-DM (BD IMag, BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Next, the enriched T lymphocytes were incubated with anti-CD8α, anti-Ly49C/F/I/H and anti-CD44 antibodies for 30 min at room temperature followed by sorting for CD8$^+$CD44$^+$Ly49$^+$ cells on a BD FACSAria cell sorter (BD Bioscience). A subset of CD4$^+$ T cells from the splenocytes of OT2 mice were then enriched by negative bead selection using the CD4$^+$ T Lymphocyte Enrichment Set-DM (BD IMag, BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's instructions. The enriched cell population contained >90%. CD4$^+$ T lymphocytes as determined by cell surface staining and flow cytometry.

2.7. Analysis of cell proliferation by CFSE staining

CD4$^+$ T lymphocytes from the spleens of six-week-old OT2 mice were labeled with CellTrace™ CFSE Cell Proliferation Kit (Invitrogen, Eugene, OR) according to an originally published protocol [22]. CD4$^+$ T cells (1×10^5) were then cultured in 200 μL RPMI 1640 medium supplemented with 10 mM HEPES, 1% penicillin, 1% streptomycin, 10% fetal calf serum, and 50 μM 2-mercaptoethanol together with 0.1 μg/mL $OVA_{323\text{-}339}$ peptide and 1×10^4 mitomycin C inactivated bone marrow-derived dendritic cells (BMDCs). Then, various ratios (from 0:1 to 2:1) of CD8$^+$CD44$^+$Ly49$^+$ T cells were co-cultured for 72 h. Finally, cells (n = 3) were analyzed by flow cytometry. The average number of cell divisions and the proportion of dividing cells were calculated using algorithms within FlowJo software.

2.8. Analysis of cell apoptosis by the Annexin V/PI assay

Apoptosis of the responding CD4$^+$ T cells was detected using the Annexin V-FITC/PI apoptosis assay kit (Multi Sciences, Hangzhou, China) according to the manufacturer's protocol. Briefly, cells were pooled together, washed with ice-cold PBS, and then re-suspended in 400 μL binding buffer. Cells were then incubated with 5 μL Annexin V-FITC and 5 μL of PI for 15 min in the dark at room

Table 1
Peptides used for the immunization.

	Sequence	Length	Origin	MHC restricted
$EphA2_{682\text{-}689}$	VVSKYKPM	9AA	Self-origin	MHC Class I(H2K^b)
$OmpC_{132\text{-}140}$	RNTDFFGL	8AA	Bacterial-origin	
$SV9_{324\text{-}332}$	FAPGNYPAL	9AA	Viral-origin	
$MOG_{35\text{-}55}$	MEVGWYRSPFSRVVHLYRNGK	21AA	Self-origin	MHC Class II (I-A^b)
$Ag85B_{240\text{-}254}$	FQDAYNAAGGHNAVF	15AA	Bacterial-origin	
$GP_{61\text{-}80}$	GLNGPDIYKGVYQFKSVEFD	20AA	Viral-origin	