Splenic dendritic cells from antigen-fed mice induced antigen-specific T cell unresponsiveness *in vivo*

Masanobu Hibi^{1,2}, Satoshi Hachimura^{2,*}, Toshiko Somaya², Etsuko Toda², Masaaki Hashiguchi², Tsuyoshi Takayama¹, Kastumi Sasaki¹, Takashi Senga¹, Shuichi Hashizume¹ and Shuichi Kaminogawa²

¹Research Center, Morinaga & Co., Ltd., Yokohama 230-8504, Japan; ²Department of Applied Biological Chemistry, University of Tokyo, Tokyo 113-8657, Japan (*Author for correspondence: E-mail: ahachi@mail.ecc.u-tokyo.ac.jp; fax: +81-3-5841-8029)

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Abstract

While it is well-known that orally fed antigens induce systemic T cell tolerance (oral tolerance), the mechanism by which this occurs, however, remains unclear. In the present study, we examined the role of splenic dendritic cells (DCs) in the process of oral tolerance induction and/or maintenance, by using an adoptive transfer system of antigen (Ag)-specific CD4⁺ T cells from ovalbumin (OVA)-specific T cell receptor transgenic mice and DCs from OVA-fed BALB/c mice. Transfer of splenic DCs from OVA-fed mice reduced IL-2 productivity and the proliferative activity of pre-transferred Ag-specific CD4⁺ T cells to *ex vivo* Ag stimulation. There were no changes in expression levels of costimulatory molecules on DCs from OVA-fed mice. Our results show that orally administered Ags induce systemic T cell unresponsiveness through splenic DCs without inducing cell division of T cells, thus providing evidence that splenic DCs are involved in oral tolerance induction.

Abbreviations: Ab – antibody; Ag – antigen; APC – antigen presenting cell; CFSE – 5,6-carboxy succinimidyl fluoresceine ester; DC – dendritic cell; EDTA – ethylenediaminetetraacetic acid; ELISA – enzyme-linked immunosorbent assay; FITC – fluorescein isothiocyanate; GALT – gut-associated lymphoid tissue; HBSS – Hank's balanced salt solution; IL – interleukin; mAb – monoclonal antibody; MHC – major histocompatibility complex; OVA – ovalbumin; PBS – phosphate-buffered saline; TCR – T cell receptor; Tg – transgenic; TGF – transforming growth factor

Introduction

It is a well-established phenomenon that soluble proteins delivered through the gastrointestinal route do not induce a systemic immune response, but induce immune tolerance (Weiner et al. 1994; Mowat and Viney 1997). This phenomenon, called oral tolerance, was first described about 90 years

ago (Wells 1911); however, the mechanisms of its induction and maintenance are not fully understood. Apart from the elucidation of its mechanism, the possibility of a clinical application has been extensively examined. It was reported that orally fed antigens (Ags) suppressed autoimmune diseases (Faria and Weiner 1999) or allergic diseases (Hirahara et al. 1998) in experimental animal

models. Moreover, it has been tested in humans with autoimmune diseases such as multiple sclerosis (Weiner et al. 1993) and rheumatoid arthritis (Trentham et al. 1993).

Ag presented by antigen presenting cells (APCs), including B cells, dendritic cells (DCs) and macrophages, are able to determine a T cell's status, such as its state of activation or unresponsiveness. DCs are reported as being the most immune stimulatory APCs that are able to prime naive T cells. On the other hand, it has been suggested that DCs can mediate tolerance. In a recent report, a subpopulation of DCs possessing self-Ags in the form of apoptotic intestinal epithelial cells in peripheral tissue was shown to migrate to T cell areas of draining lymph nodes, which may be related with self-tolerance (Huang et al. 2000). In another report, the result of Ag selectively delivered to DCs using specific antibodies (anti-mannose receptor) suggested that Ag presented by DCs induced Ag-specific T cell tolerance under steady state conditions (Hawiger et al. 2001). These reports suggested that DCs might play an important role in self-tolerance induction in peripheral tissue, and not only in the deletion of self-reactive T cells as in the case of thymic-tolerance induction.

It is possible that DCs are involved in the induction and/or maintenance of oral tolerance as well. It appears that different mechanisms of tolerance induction apply to orally administered Ag in different sites – local or systemic. Locally, in the gut-associated lymphoid tissue (GALT), orally fed Ag induces immune suppression through TGF- β and IL-10 productions from T cells (Gonnella et al. 1998). It has been suggested that gut-derived DCs may mediate this response (Alpan 2000). On the other hand, it has been well described that the capacity to proliferate and to secrete IL-2 in response to Ag is reduced in systemic T cells (termed unresponsiveness or anergy) after oral administration of Ags (Melamed and Friedman 1993). It has been demonstrated that gut-absorbed proteins enter the blood stream rapidly and can induce the suppression of systemic delayed-type hypersensitivity (Peng et al. 1990). However, the organ or site where orally absorbed soluble Ag induces systemic tolerance, and the role of DCs in this process remain undefined.

The aim of this study was to identify the role of splenic DCs in the induction of systemic immune tolerance when Ag is orally administered. We used

the adoptive transfer system of Ag-specific T cells, in which OVA-specific T cells from T cell receptor (TCR) transgenic (Tg) mice are transferred into syngeneic BALB/c mice (Van Houten and Blake 1996). In this system, it has been demonstrated that orally fed Ag could reduce IL-2 production and proliferation of the adoptively transferred Ag-specific T cells. In the present study, DCs from OVA-fed mice were transferred into the recipient mice which had received the OVA-specific T cells.

Materials and methods

Mice

Female BALB/c mice (6–10 weeks of age) were obtained from Clea Inc. (Tokyo, Japan). Female DO11.10 OVA-specific TCR Tg mice (DO11.10 mice) on a BALB/c background and originally established by Murphy et al. (1990) were used at 7–12 weeks of age. All experiments were performed in accordance with the guidelines of laboratory animals of The University of Tokyo.

Cell preparation and CFSE staining

Naive CD4⁺ T cells were prepared from naive DO11.10 mice. Spleen cells were digested with collagenase D (1 mg/ml, Roche, Mannheim, Germany), and DNase I (0.1 mg/ml, Roche) at 37 °C for 30 min, and further dissociated in Ca²⁺-free HBSS in the presence of 5 mM EDTA at 37 °C for 5 min. CD4⁺ T cells were positively sorted by MACS using anti-CD4 microbeads (GK1.5, Miltenyi Biotec, Bergish Gladbach, Germany). Cell purity was checked by flow cytometry (FACsort, BD Biosciences, San Diego, CA) and the percentage of CD4 positive T cells was found to be more than 95%. In some experiments, CD4⁺ T cells obtained by MACS were labeled with 5, 6 carboxy succinimidyl fluoresceine ester (CFSE, Molecular probes, Eugene, OR). CD4⁺ T cells were suspended at 2 μ M CFSE in PBS and incubated for 10 min at RT, and after incubation, cells were washed three times and resuspended in PBS. To prepare CD11c⁺ DCs, fresh spleen cells from naive BALB/c mice were digested and dissociated as described above. After blocking of Fc receptors (purified anti-mouse CD16/32 mAb, 2.4G2, pre-

pared in our laboratory), single cells were sorted by MACS using anti-CD11c microbeads (N418, Miltenyi Biotec). For peptide pulse, DCs were isolated from naive BALB/c mice and cultured in medium containing 500 nM of OVA peptides for 2 h.

Adoptive transfer

For the transfer of KJ1.26⁺ T cells into BALB/c mice, naive CD4⁺ T cells (5×10^6 cells) obtained from spleens of DO11.10 mice, stained by CFSE, and resuspended in PBS, were i.v. injected into syngeneic BALB/c mice. After 1 day, 5×10^6 CD11c⁺ DCs were injected intravenously.

Oral administration

BALB/c mice were fed with 250 mg of OVA (2× crystallized, Seikagaku-kogyo, Tokyo, Japan) in PBS by intragastric gavage at 6 and 18 h before anatomy. Control mice received PBS.

Flow cytometry

The following antibodies (Abs) were used for identification of cell populations and phenotype analysis: APC-conjugated anti-mouse CD4 monoclonal Ab (mAb) (H129.19, Pharmingen), PE-conjugated anti-mouse TCR clonotype mAb (KJ1.26, Caltag, South San Francisco, CA), APC-conjugated anti-mouse CD11c mAb (HL3, Pharmingen), FITC-conjugated anti-mouse CD45R/B220 (RA3-6B2, Pharmingen), biotin-conjugated anti-mouse CD80 mAb (16-10A1, Pharmingen), biotin-conjugated anti-mouse CD86 mAb (GL1, Pharmingen), biotin-conjugated anti-mouse CD40 mAb (3/23, Pharmingen), and PE-conjugated streptavidin (Pharmingen). Analysis was performed with a FACSort flow cytometer (BD Biosciences).

Cell culture and proliferation assay

Spleen cells (5×10^5) were stimulated with indicated concentrations of OVA_{323–339} (OVA peptide; ISQAVHAAHAEINEAGR, Mitokor, San Diego,

CA) in 0.2 ml of RPMI-1640 medium supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, 100 U/ml penicillin, and 100 µg/ml streptomycin in 96-well culture plate. The cells were incubated at 37 °C in a CO₂ incubator. Culture supernatants were collected at 24 h for quantification of IL-2 concentration by ELISA. Proliferation was measured by pulsing 1 µCi of [³H]thymidine per well during the last 24 h of culture. The proliferative response was expressed as cpm per KJ1.26 positive cells.

IL-2 ELISA

The collected culture supernatants were determined for their IL-2 concentration by ELISA. A Maxisorp immunoplate (Nunc, Roskilde, Denmark) was coated with purified anti-mouse IL-2 capture antibody (JES6-1A12, Pharmingen). After blocking with bovine serum albumin (Seikagaku Kogyo), diluted samples and mouse IL-2 standards were applied to the plate. Bound antibody was detected using biotinylated anti-mouse IL-2 mAb (JES6-5H4, Pharmingen), streptavidin-alkaline phosphatase conjugate (Zymed, South San Francisco, CA), and the substrate of *p*-nitrophenylphosphate disodium salt.

Statistics

Differences between mean values were analyzed by Student's *t* test.

Results

DCs from Ag-fed mice do not induce significant cell division of Ag-specific CD4⁺ T cells in vivo

We investigated whether DCs from Ag-fed mice could stimulate Ag-specific T cells to proliferate *in vivo*. For the analysis of cell division of Ag-specific CD4⁺ T cells stimulated by MHC class II-peptide complexes on DCs, naive CD4⁺ T cells were obtained from spleens of DO11.10 mice and stained by CFSE. CFSE is a fluorescein-based dye that labels molecules within living cells, and it can be used to track cell division due to the progressive

Figure 1. Analysis of cell division of KJ1.26⁺ CD4⁺ T cells stimulated by DCs. Naive CD4⁺ T cells were obtained from spleens of DO11.10 mice and stained with CFSE. These CD4⁺ T cells were i.v. injected into syngeneic BALB/c mice, and after 1 day, CD11c⁺ DCs were injected intravenously. On day 7 after DC transfer, the percentage and cell division of KJ1.26⁺ CD4⁺ cells were analyzed using anti-clonotype mAb. Fluorescence intensity of CFSE was analyzed after gating for KJ1.26⁺ and CD4⁺ cells. (a) DCs were isolated from naive BALB/c mice and were cultured in medium containing 500 nM of OVA peptide for 2 h (pep-DC) before transfer. (b,c) DCs were isolated from BALB/c mice fed with 250 mg of OVA in PBS i.g. at 6 and 18 h before anatomy (Tg-T cell/OVA-DC) or control mice that received PBS i.g. at 6 and 18 h before anatomy (Tg-T cell/PBS-DC). (b) The percentage of KJ1.26⁺ CD4⁺ T cells in the spleen. (c) Cell division of Ag-specific T cells was visualized by gating on KJ1.26⁺ CD4⁺ cells and analyzing CFSE fluorescence intensity by flow cytometry.

halving of the fluorescence intensity of dye in cells after each division (Lyons and Parish 1994). The CD4⁺ T cells were i.v. injected into syngeneic BALB/c mice, and after 1 day, CD11c⁺ DCs were injected intravenously. On day 7 after DC transfer, the percentage of OVA-specific CD4⁺ T cells was analyzed using anti-clonotype mAb KJ1.26. We first confirmed that cell division of Ag-specific T cells could be induced by Ag presentation of transferred DCs in this system. We detected vigorous cell division of KJ1.26⁺ CD4⁺ T cells (OVA-specific T cells) *in vivo*, by injected DCs that were pre-pulsed *in vitro* with 500 nM of OVA

peptide, which is demonstrated by diminished CFSE fluorescence of these cells (Figure 1a). Next, when we transferred splenic DCs from OVA-fed mice (OVA-DCs) and/or DCs from control PBS-fed mice (PBS-DCs), we could detect a small population of KJ1.26⁺ CD4⁺ T cells, similar in size, in both the OVA-DC group and control (PBS-DC) group (Figure 1b). Cell division of Ag-specific T cells was visualized by gating on KJ1.26⁺ CD4⁺ cells and analyzing CFSE fluorescence intensity by flow cytometry. There was little difference between OVA-DC transferred mice and control DC (PBS-DC) transferred mice in

patterns of KJ1.26⁺ CD4⁺ T cell division (Figure 1c). Thus, cell division of adoptively transferred Ag-specific T cells was not elicited by DCs from Ag-fed animals.

The proliferation of adoptively transferred KJ1.26⁺ CD4⁺ T cells is significantly reduced when DCs from OVA-fed mice are cotransferred

Next, we asked whether splenic DCs from OVA fed mice had the ability to induce systemic tolerance of i.v. transferred KJ1.26⁺ CD4⁺ T cells. Splenocytes were isolated from BALB/c mice which had been injected with 5×10^6 TCR Tg CD4⁺ T cells and same number of DCs from OVA-fed BALB/c mice or control PBS-administered mice, and were then cultured in the presence or absence of 500 nM OVA peptide for 4 days. [³H]Thymidine was added for the last 24 h of culture to measure the proliferative response. The [³H]thymidine incorporated was calculated per cell number of OVA-specific KJ1.26⁺ CD4⁺ cells. The results shown in Figure 2 demonstrated that the injection of DCs from OVA-fed mice (OVA-DCs) significantly ($p < 0.01$) reduced the proliferative potential of OVA-specific KJ1.26⁺ T cells in their response to *ex vivo* stimulation of OVA peptide, compared with the injection of DCs from control mice (PBS-DCs). T cell proliferation without *ex vivo* Ag was also reduced significantly.

IL-2 production of adoptively transferred KJ1.26⁺ CD4⁺ T cells is significantly reduced when DCs from OVA-fed mice are cotransferred.

IL-2 production by splenocytes, which were isolated from chimeric BALB/c mice injected with KJ1.26⁺ CD4⁺ T cells and OVA-DCs or control PBS-DCs, was measured after stimulation with OVA peptide for 24 h. As expected, KJ1.26⁺ CD4⁺ T cells from mice which injected with OVA-DCs showed significantly ($p < 0.01$) reduced IL-2 production in response to *ex vivo* Ag stimulation compared to T cells from control PBS-DC-injected mice (Figure 3). By using MACS sorted CD4⁺ cells from spleen in the presence of APCs (mitomycin C-treated BALB/c splenocytes), we obtained similar results (data not shown). IL-2 production from T cells without *ex vivo* Ag stimulation was also reduced significantly in mice injected with OVA-DCs.

Figure 2. Proliferation of Ag-specific T cells in the spleen of BALB/c mice which received DO11.10 CD4 T cells and DCs. BALB/c mice were intravenously injected with 5×10^6 CD4⁺ T cells. One day after, mice were injected i.v. with 5×10^6 DCs from OVA-administrated (OVA-DC) or PBS-administrated (PBS-DC) BALB/c mice. After 7 days, cells from spleen were cultured with or without 500 nM OVA peptide for 96 h. One μ Ci of [³H]thymidine was added for the last 24 h of culture to measure the proliferative responses of these cells. Proliferation was expressed as per cell number of KJ1.26⁺ cells. *, $p < 0.01$ versus PBS-DC injected group. Similar results were obtained in two independent experiments.

Figure 3. IL-2 production of Ag-specific T cells in the spleen of BALB/c mice which had received DO11.10 CD4⁺ T cells and DCs. BALB/c mice were intravenously injected with 5×10^6 CD4⁺ T cells. One day after, mice were injected i.v. with 5×10^6 DCs from OVA-administrated (OVA-DC) or PBS-administrated (PBS-DC) BALB/c mice. After 7 days, splenocytes were cultured with or without 500 nM OVA peptide. At 24 h, the IL-2 levels of culture supernatants were determined by sandwich ELISA. Results were calculated per KJ1.26⁺ cells. *, $p < 0.01$ versus PBS-DC injected group. Similar results were obtained in three independent experiments.

Figure 4. To characterize the DCs from OVA-administered BALB/c mice splenocytes (OVA-DC), we compared the cell surface expression of various molecules on OVA-DCs with those of control splenic DCs derived from PBS-fed mice (PBS-DC). DCs were analyzed by flow cytometry to visualize cell surface expression of CD80, CD86 and CD40 molecules after gating on CD11c⁺ B220⁻ cells.

The expression of cell surface molecules on DCs is not altered by OVA feeding

To characterize the DCs in OVA-fed mice (OVA-DCs), we compared the cell surface expression of various molecules on splenic DCs from OVA-fed BALB/c mice with control PBS-fed mice (PBS-DCs) (Figure 4). DCs from OVA-fed mice and control mice were analyzed by flow cytometry to visualize surface molecules after sorting by MACS. The expressions of CD80, CD86 and CD40 were at similar levels. Moreover, we could not observe any significant differences in the expression levels of surface molecules such as MHC class II, CD80, CD86, CD40 in a long term feeding (10% OVA containing diet, 2 weeks) experiment (data not shown).

Discussion

In the present study, splenic DCs from OVA-fed mice were transferred to mice which had received OVA-specific TCR Tg T cells in order to elucidate

the role of splenic DCs in the induction of oral tolerance. Although we were able to observe *in vivo* T cell division that was stimulated by MHC–Ag complexes presented on DCs that acquired Ag *in vitro* by employing the CFSE system, we could not detect KJ1.26⁺ CD4⁺ T cell division caused by OVA peptides presented by MHC class II molecules on DCs from OVA-fed mice in the spleen (Figure 1), mesenteric lymph nodes or peripheral lymph nodes (data not shown). We do not consider that Ag was not presented on DCs, but rather, we consider that oral Ag presented by DCs did not lead to T cell division. Gutgemann et al. (1998) demonstrated that Ag-specific splenic T cells were rapidly activated and tolerized by systemic presentation of an orally administered Ag. They found that three feedings of cytochrome *c* (cyt *c*) activated cyt *c*-specific T cells in the spleen and mesenteric lymph nodes 6 h later, as determined by CD69 up-regulation and a decrease in T cell proliferation and IL-2 production. This suggested that orally administered Ags are presented in peripheral lymphoid tissue. In this study we observed in the induction of Ag-specific

unresponsiveness after transfer of DCs from OVA-fed mice (Figures 2 and 3). Taken together, it appears unlikely that the amount of antigen presented on OVA-fed DCs was insufficient to be recognized by CD4⁺ T cells in our adoptive transfer system. There is some possibility that only a small quantity of oral antigen reached the spleen and was presented at low levels on splenic DCs. Indeed it was suggested that DCs presenting low levels of antigen induced T cell tolerance (Kawahata et al. 2002). In a study using self-Ag expressing mice, CD4⁺ T cell tolerance induction has been shown to be independent of cell division (Adler et al. 2000). The results of our experimental model point to the hypothesis that tolerance inducible DCs might mediate Ag-specific T cell tolerance without promoting cell division.

It has been demonstrated that DCs play a central role in inducing peripheral unresponsiveness in self-tolerance or mucosal tolerance using adoptive transfer systems. In an experimental system for airway administration of Ags, pulmonary DCs exposed to Ag produced IL-10 and, as a result, could induce low responsiveness of T cells (Akbari et al. 2001). In *t* experimental allergic encephalomyelitis model, transferred CD8⁻ CD4⁺ DCs mediated both unresponsiveness and bystander suppression in T cells (Legge et al. 2000). We asked whether splenic DCs from OVA-fed mice induced Ag-specific T cell tolerance in this adoptive transfer system. Splenic DCs from mice fed with OVA reduced both proliferative activity (Figure 2) and IL-2 productivity (Figure 3) of the T cell response to *ex vivo* Ag. Our data demonstrated that splenic DCs could induce systemic T cell tolerance. However, we could not find any changes in the expression of CD80, CD86 and CD40 on DCs (Figure 4). There is the possibility that only a minor population of DCs presented the Ag, which showed altered cell surface expression of these costimulatory molecules, and these cells could induce Ag-specific T cell tolerance. We found that the responses of cells in the absence of *ex vivo* Ag stimulation including IL-2 production and cell proliferation were also significantly decreased. It is possible that the interaction between DCs from OVA-fed mice (which presented the Ag) and OVA-specific CD4 T cells induced suppression in the absence of additional Ag.

In contrast to splenic T cells, the proliferation and IL-2 production by T cells in mesenteric

lymph nodes and other peripheral lymph nodes were not reduced (data not shown). Lappin et al. demonstrated that DCs injected intravenously homed to the spleen, liver and lung, but not to peripheral lymph nodes after 24 h (Lappin et al. 1999). In our study, it is likely that we could not detect T cell tolerance in local lymph nodes, because i.v. transferred DCs could not reach mesenteric lymph nodes or other peripheral lymph nodes.

In conclusion, we have shown that orally administered Ags induce systemic T cell unresponsiveness through splenic DCs without inducing cell division of T cells. Further studies addressing whether OVA-fed DCs produce suppressive cytokines or induce regulatory T cells such as CD25⁺ CD4⁺ T cells, and the identification of the DC subsets which present oral antigen will be of significant interest. Such studies of DCs presenting oral antigen should help to reveal unexplained characteristics of the immune system, and provide clues for understanding the complexities of cellular responses in the body.

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