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Oral tolerance and OVA-induced tolerogenic dendritic cells reduce the severity of collagen/ovalbumin-induced arthritis in mice

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ABSTRACT

Dietary proteins play an important role in the regulation of systemic immune response, in a phenomenon known as oral tolerance (OT). To evaluate the effects of OT on a murine model of type II collagen (CII) plus ovalbumin (OVA)-induced arthritis (CIA), mice were fed with OVA either before or after CIA induction. OT significantly reduced the paw edema and synovial inflammation, as well as serum levels of anti-CII, the ex vivo proliferation and inflammatory cytokine production by spleen cells from CIA mice. The frequencies of Foxp3$^+$ and IL-10$^+$ cells were higher, whereas IFNγ^+ cells and IL-17$^+$ cells were lower, among gated CD4$^+$ spleen T cells from tolerized CIA mice than in those from non-tolerized CIA mice. Adoptive transfer of tolerogenic dendritic cells (DCs) before CIA induction mimics the effects observed in the OT. We demonstrate here that bystander suppression induced by OT can modify the course of CIA and tolerogenic DCs play a role this phenomenon.

1. Introduction

Human rheumatoid arthritis is a chronic inflammatory autoimmune disease characterized by pain, swelling and deformity of the joints. The immune response in arthritis involves the activation of antigen-specific lymphocytes secreting inflammatory cytokines that act on macrophages, osteoclasts and cells adjacent to inflamed synovial tissue [1]. Collagen-induced arthritis (CIA) is the most studied experimental model of rheumatoid arthritis, which involves the administration type II collagen (CII) plus complete Freund's adjuvant (CFA) in susceptible strains of mice and rats. In resistant BALB/c mice, however, CIA is only observed when a second protein, such as keyhole-limpet hemocyanin (KLH) or ovalbumin (OVA), is combined to the CII and CFA [2].

The use of knockout animals and blockage with monoclonal antibodies has shown that IL-17, IFN-γ and TNF-α play a fundamental role in the establishment of arthritis [3–5]. Monoclonal antibody based therapies have been proved to be an alternative to the classic use of immunosuppressive disease-modifying antirheumatic drugs [6], with rare adverse effects in humans [7,8].

Oral tolerance (OT) has been successfully used to alter the course of inflammatory responses in some experimental autoimmune diseases [9,10]. Joint inflammation, for example, has been significantly reduced in mice tolerized by repeated oral feeding of CII before CIA induction [9]. The tolerized mice also exhibited an increased serum IgG1, a reduction of serum IgG2a, a suppression of specific proliferative response of T cells from lymph nodes, and an increased frequency of cells producing IL-10 amongst T CD4$^+$CD25$^+$ splenic cells stimulated with CII.

Dendritic cells (DCs) play an important role in immune responses of T lymphocytes that occur in the microenvironment of digestive tract mucosa, directing them to either an effector profile or systemic tolerance to the antigen. The main effect induced by DCs in immune tolerance seems to be the expansion of TGF-β- and IL-10-secreting regulatory T lymphocytes (Tregs) that inhibit the development of IFN-γ-, TNF-α- and IL-17-secreting T cells [9–11]. CD103$^+$ DCs in small intestine exhibit an enhanced ability to metabolize vitamin A and generate its major active metabolite retinoic acid [12]. It has been suggested that retinoic acid underlies the CD103$^+$DC capacity to induce the gut homing receptors CC chemokine receptor (CCR)9 and α4β7 on responding T and B cells, to enhance transforming growth factor (TGF)β-dependent naïve T cell conversion to forkhead box (Fox)P3$^+$ T regulatory cells (Tregs) while suppressing TGFβ-dependent Th17 cell differentiation [13,14].

However, currently it is unknown whether OT to ovalbumin is capable of reducing clinical signs of CIA in BALB/c mice. In this

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study, we show that administration of OVA by oral route reduced the severity of CIA in BALB/c mice, and that this phenomenon occurred when OVA is administered either before or after the onset of the disease. Our data also suggested that bystander suppression induced by the ingestion of OVA leads to the generation of tolerogenic DCs with the consequent increase of the proportion of $CD25^{+}Foxp3^{+}$ Tregs and secretion of anti-inflammatory cytokines in arthritic mice.

2. Material and methods

2.1. Animals

Eight weeks old male BALB/c mice from the Multidisciplinary Center for Biological Research, University of Campinas (UNICAMP), were used in this study. The animals were kept in *specific-pathogen free* condition, in a controlled temperature and photoperiod environment, with autoclaved food and water *ad libitum* throughout the experiment. The protocols involving laboratory animals were approved by the Institutional Committee (Comissão de Ética no Uso de Animais – CEUA/UNICAMP; Protocol number 1759-1).

2.2. Oral tolerance

The induction of OT to OVA followed the protocol previously described [15]. Briefly, 4 mg/mL OVA (Sigma–Aldrich, St. Louis, MO, USA) were added to water supply, for a week. After this period, the liquid supply was replaced by protein-free water. Three animals in this group were challenged with two doses of 10 μg OVA plus 1 mg $AlOH_3$ at intervals of 1 week to evaluate the induction of OT. Low levels of anti-OVA detected in sera obtained from these animals indicated the success of the procedure adopted.

2.3. Type II collagen-induced arthritis

Arthritis was induced in male mice as described elsewhere [2]. Briefly, chicken type II collagen (kindly provided by Dr. Benedito dos Campos Vidal, UNICAMP) and OVA were emulsified in complete Freund's adjuvant (CFA). Animal of naïve group received a subcutaneous (s.c.) dose of antigens constituted by mixture of 100 μg of each protein emulsified in CFA (v:v) at the base of the tail. Two additional doses of the CII + OVA in CFA were applied on days 21 and 45. Half of this group of mice was fed with OVA to induce tolerance over the days 28 and 35 after the beginning of subcutaneous immunization. A third group consisted of male mice fed OVA for 7 consecutive days prior arthritis induction. On day 65, mice of all groups were euthanized for histological and immunological analysis.

2.4. Evaluation of inflammation and histological analysis

In order to evaluate the articular edema in CIA mice, paw thicknesses were measured weekly from day 21 to 65. On day 65 after CIA induction, the animals of each experimental group were sacrificed, its hind legs were dissected, fixed and decalcified in formic acid solution 15% and formaldehyde 5%, cut in microtome (cuts of 6 μm) and stained in Sirius Red for histopathological examination.

2.5. Detection of serum antibodies

Detection of anti-OVA serum antibodies was carried out in microtiter plates covered with 20 μg/mL OVA in sodium carbonate buffer, pH 9.0, as described elsewhere [15]. To detect anti-type II collagen serum antibodies, plates were covered with 20 μg/mL chicken type II collagen (Chondrex Inc, Redmond, WA, USA) in phosphate buffered saline (PBS) pH 7.2 as described elsewhere [16]. Absorbance was read at 492 nm in an ELISA reader (Labsystem MS, Finland). The results of ELISA were expressed as averages of sum ± standard error of mean (S.E.M.) of the optical densities obtained in each experimental group.

2.6. Co-culture experiments

Dendritic cells were isolated from spleens of naïve, immunized and OVA-tolerant mice employing anti-CD11c magnetic beads (Miltenyi Biotech, Auburn, CA, USA) following the manufacturer's recommendations. After purification procedures, about 80% of the cells were MHC II^{+} and only 3% were $CD3^{+}$. $CD11c^{+}$ DCs at concentration of 1×10^{6}cells/mL were pulsed for 18 h with 50 μg/ml of CII. Splenic cells from arthritic mice were enriched in T lymphocytes using nylon wool column, according to protocol previously described [15]. T lymphocytes were stained with carboxyfluorescein succinimidyl ester (CFSE) according to manufacturer's recommendations (Sigma), and cultured in 96-well plates at a concentration of 2×10^{5}/well in the presence of pulsed DCs at a ratio of 10:1 (T:DC) with 50 μg/ml CII for 96 h. Before the addition of antigen, an aliquot of stained cells was withdrawn for analysis by flow cytometer to define the maximum value of incorporation of the probe. Supernatants were collected for dosage of cytokines through ELISA. To measure proliferation, cells were fixed with PBS containing 1% formaldehyde, transferred to appropriate tubes and analyzed in flow cytometer. To examine expansion of subsets of T lymphocytes, aliquots were processed to flow cytometry.

2.7. Measurement of cytokines

At the end of the lymphocyte cultures, the supernatants were collected for dosage of cytokines through ELISA, using commercial kits according to manufacturer's recommendations. It was analyzed: IL-12, TNF-α (BD PharMingen), IL-17, IL-6, IFNγ, IL-10, IL-4 and TGF-β (e-Bioscience).

2.8. Adoptive transfer experiments

2.8.1. Dendritic cell purification and transfer

Splenic dendritic cells were isolated from naïve and OVA-tolerant mice employing anti-CD11c magnetic beads (Miltenyi Biotech), according to the manufacturer's recommendations. Cells were pulsed with OVA 50 μg/mL during 18 h and adoptively transferred to naïve mice via retro-orbital plexus, in three doses of 5×10^{5} cells per animal on days 6, 4 and 2 before the CIA induction.

2.8.2. Spleen cell proliferation

On day 65 after CIA induction, mice of all groups of the adoptive transfer experiments were killed and spleens were aseptically removed. Splenocytes were stained with CFSE, seeded at 2×10^{5} cells/well in the presence of 50 μg/mL CII, and then incubated for 96 h at 37 °C in humidified incubator with CO2 5%. Cells cultured in absence of stimuli were used as baseline control. Supernatants were collected for dosage of cytokines. The cells were fixed with PBS containing 1% formaldehyde and proliferation was assessed in $CD3^{+}CFSE^{low}$ by flow cytometry. The results were expressed as proliferation index (fold change), calculated by dividing the percentages found in the experimental groups by the percentage found in the control group.

2.9. Flow cytometry

Following cultivation or in vivo treatments, single cell suspensions were washed and suspended in staining buffer (PBS plus