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Oral or Nasal Antigen Induces Regulatory T Cells That Suppress Arthritis and Proliferation of Arthritogenic T Cells in Joint Draining Lymph Nodes

Femke Broere,1* Lotte Wieten,* Elles I. Klein Koerkamp,* Joel A. G. van Roon,† Teun Guichelaar,* Floris P. J. G. Lafeber,‡ and Willem van Eden*  

The propagation of mucosal tolerance as a therapeutic approach in autoimmune diseases remains a difficult goal to achieve, and therefore further mechanistic studies are necessary to develop potential clinical protocols to induce mucosal regulatory T cells (Tr cells). In this study we addressed whether oral or nasal proteoglycan induced functional Tr cells in the cartilage proteoglycan-induced chronic arthritis model. Both nasal and oral application of human proteoglycan before induction of disease suppressed arthritis severity and incidence. Tolerized mice showed enhanced numbers of IL-10 producing CD4+ cells in the paw-draining lymph nodes. Furthermore, CD4+ spleen cells displayed enhanced expression of molecules associated with Tr cells, such as IL-10, Foxp3, and TGF-β. Transfer of CD4+ spleen cells from mucosally tolerized donors into proteoglycan-immunized mice abolished arthritis and reduced humoral responses, indicative of Tr cells with the capacity to inhibit already induced immune responses. Tr cells were activated upon transfer, because enhanced proliferation was observed in the joint draining lymph nodes compared with activated T cells from nontolerized donors. Upon cotransfer with naive proteoglycan-specific T cells, mucosally induced Tr cells inhibited proliferation of these arthritogenic T cells in vivo. Herein we show that both oral and nasal Ag application induced Tr cells, which had a direct inhibitory effect on already established pathogenic B and T cell responses. The Journal of Immunology, 2008, 181: 899–906.

Mucosal tolerance is important to prevent inadequate and pathogenic reactivity to normally innocuous Ags that enter the body via mucosal surfaces. In animal models of inflammatory diseases, both oral and nasal Ag application has been shown to protect against the induction of disease via the induction of regulatory T cells (Tr cells)1,2 (1, 2). Mucosal tolerance has been proposed as a means to induce Ag-specific protection in inflammatory diseases, ranging from autoimmune disease to transplant rejection and allergy (3, 4). However, successful translation of prevention of disease in animal models to therapeutic application in human disease proved to be difficult (5–11). The disappointing outcome of clinical trials can be explained by several facts, such as: the trials were performed in end-stages of disease; the patient populations studied were inherently diverse; and there was a lack of pretreatment screening to determine whether the Ag was immunologically relevant in patients to be treated.

Despite these difficulties, it is unequivocal that oral tolerance can be achieved in humans (12–17). In some cases, Ags fed suppressed in vitro T cell recall responses but failed to suppress Ag-specific IgG, IgM, and secretory IgA responses, suggesting a differential effect on T and B cell responses (12). Intriguingly, this seems not to be the case in IgE-mediated allergy, as it was shown that desensitization by oral immunotherapy was highly successful (18).

Previously, it was demonstrated that relevant epitopes of the cartilage proteoglycan (PG) were recognized by T cells in the context of human class II MHC in HLA-DR4- and HLA-DQ8-transgenic (Tg) mice (19). Moreover, several studies in rheumatoid arthritis (RA) patients showed T and B cell responses against the human PG, indicating that PG may be a potential autoantigen in RA (20–22). Collectively, these studies suggest that PG may be a target of disease-associated T cell responses in patients with RA (23–26) (S. E. Berlo, H. de Jong, W. van Eden, and B. J. Prakken, unpublished results). Difficulties with translating animal models into clinical trials clearly show the need to further unravel the complex mechanisms of mucosal tolerance, and to explore their application in experimental autoimmune disease models. Because orally and nasally induced tolerance might induce different Tr cells (27) we wanted to explore both forms of tolerance induction in the PG-induced arthritis (PGIA) model. PGIA is a chronic relapsing model for RA, and disease is based on a combined T and B cell response directed against joint cartilage PG (28–30).

In this model we studied the potential regulatory role of mucosally induced Tr cells and whether these cells could alter the in vivo response of potentially arthritogenic T cells.

Oral and nasal application of PG was found to suppress the induction of disease in a comparable fashion. The reduction of arthritis severity correlated with enhanced numbers of IL-10-producing Ag-specific CD4+ T cells in the local draining lymph nodes. Additionally, enhanced mRNA levels for IL-10 and Foxp3 in CD4+ splenocytes indicated the presence of increased numbers of functional Tr cells within this T cell pool, as also shown by reduced arthritis severity in acceptor mice upon CD4+ spleen cell
transfer. Furthermore, transfer of mucosally induced Tr cells led to IL-10 production in the joint draining lymphoid tissues and reduced influx of CD4+ cells and neutrophils into the joints. In the same experiments, transferred (CFSE-labeled) mucosally induced CD4+ Tr cells exhibited enhanced proliferation in the joint draining lymph nodes and inhibited proliferation of naïve arthritogenic T cells in vivo.

Materials and Methods

Mice

Female BALB/c retired breeder mice, aged between 16 and 26 wk, were purchased from Charles River Laboratories. PG-TCR Tg mice were bred and kept under standard conditions (31, 32). Animals were routinely housed and received water and chow ad libitum. Experiments were approved by the Animal Experimental Committee of the University of Utrecht.

Abs and Ag

Anti-CD8 (53-672), anti-CD11b (M1/70, MAC-1), anti-F4/80, anti-MHC class II (M5/114), and anti-CD45R (6B2) were used as culture supernatants at predetermined optimal concentrations. PE-conjugated anti-CD69 (H1.2F3), anti-CD27 (Lg.3A1), and anti-CD25 (PC61) were purchased from BD Pharmingen.

Proteoglycan (PG) (aggrecan) was purified from human articular cartilage by 4 M guanidinium chloride extraction, and depleted of glycosaminoglycan side chains using endo-β-galactosidase (0.22 mM/mg dry weight) and testicular hyaluronidase (5 U/mg dry weight) as described before (13, 14). For intranasal treatment, potentially present contaminating endotoxins were removed from PG by a Triton X-114 gradient. After this, the PG was extensively washed and a Limulus amebocyte lysate assay was performed according to the manufacturer’s protocol to test for endotoxin contamination. Endotoxin levels were <50 EU/mg.

Tolerance induction and arthritis induction

Mice were either tolerized with 3x100 μg endotoxin-low PG intranasally (i.n.) in 10 μl PBS or 3 x 1 μg PG via gavage in 200 μl PBS on days −7, −5, and −3. Control mice received OVA grade V as a control. Subsequently, mice were immunized for the induction of arthritis on days 0 and 21. Mice were injected i.p. with 400 μg PG in the synergistic adjuvant dimethyl dioctadecyl ammonium bromide (DDA; 2 mg) in 200 μl solution with PBS.

Assessment of arthritis

Paws of all mice were examined three times per week to record abnormalities due to arthritic changes of the joints. The onset and severity of arthritis were determined using a visual scoring system based on swelling and redness of paws in a blinded setup as described previously (28–30). In brief, the degree of joint swelling for each paw (scored ranging from 0 to 4) was used to express a cumulative arthritis score, with a possible maximum severity index of 16 per animal. The first clinical appearance of swelling was recorded as the onset of arthritis.

Cytokine secretion and cytokine ELISA

The percentage of cytokine-secreting cells and the amount of cytokine secreted during a 24-h restimulation period were assessed in spleen and joint draining lymphoid tissues from naive mice was used to allow normalization between experiments. Primers used were: HPRT: sense 5'-TGA AGT ACT CAT TAT AGT CAA ACC TCT CG-3' and testicular hyaluronidase (5 U/mg dry weight) as described before (13, 14). For intranasal treatment, potentially present contaminating endotoxins were removed from PG by a Triton X-114 gradient. After this, the PG was extensively washed and a Limulus amebocyte lysate assay was performed according to the manufacturer’s protocol to test for endotoxin contamination. Endotoxin levels were <50 EU/mg.

ELISA for PG-specific Abs

PG-specific Abs were measured by ELISA as described previously (28, 30, 33). ELISA 96-well plates (Corning) were coated overnight with hPG (0.1 μg protein/well) or native mPG (0.15 μg protein/well) and blocked with 1% fat-free milk in PBS. Sera were applied at increasing dilutions, and isotypes of PG-specific Abs were determined using peroxidase-conjugated mAbs to mouse IgG1 or IgG2a (BD Biosciences) as secondary Abs (33). Serum Ab levels were calculated relative to a corresponding mouse IgG isotype standards (all from BD Biosciences) or mouse serum Ig fractions (Sigma-Aldrich) (28, 30, 33).

Transfer to assess regulatory function of T cells

Single-cell suspensions from spleens of donor mice were depleted of erythrocytes in ACK lysis buffer (150 mM NH4Cl, 1 mM NaHCO3, pH 7.4) and were stained with mAbs specific for CD45R, CD11b, F4/80, MHC class II, and CD8. Positive cells were removed with sheep anti-rat-conjugated Dynal beads (Dynal Biotech).

Negative cells, denoted as enriched CD4+ T cells (purity routinely between 85 and 95%), were resuspended in PBS. Per recipient, 1x106 cells were transferred at day 20. Mice were immunized at days 0 and 21 for induction of arthritis. For tracking of CD4+ cells, we resuspended enriched CD4+ cells in PBS at 106 cells/ml and incubated these for 1 min at 37°C with CFSE (Molecular Probes) at a final concentration of 5 μM to follow their division profiles in vivo. CFSE-labeled CD4+ T cells were washed in ice-cold PBS with 2% FCS and resuspended in saline. Each mouse received 1x106 CD4+ CFSE-labeled cells in 100 μl saline by i.v. injection. For tracking of naïve CD4+ T cells isolated from PG-TCR Tg mice, cells were labeled with PKH26. Cells were incubated with 2x106 M PKH26 dye in diluent C for 5 min at 25°C to 2x106 cells/ml. Labeled cells were washed in PBS with 2% FCS and resuspended in saline.

Quantitative PCR

For quantitative analysis of mRNA expression, 1x106 cells or total joint-extracted cells were isolated and total RNA was isolated using the Qiagen RNeasy kit. Subsequently, RNA was transcribed into cDNA using the Script cDNA synthesis kit (Bio-Rad) according to the manufacturer’s protocol. Real time quantitative PCR (Q-PCR) was performed using a Bio-Rad iCycler based on (specific primers and) general fluorescence detection by SYBR Green. H Pratt and GAPDH were used as control for sample loading and to allow normalization between samples. cDNA obtained from lymphoid tissues from naïve mice was used to allow normalization between experiments. Primers used were: HPRT: sense 5’-CTG GTG AAA AGG ACC TCT CG-3’, antisense 5’-TGA ACT CAT TAT AGT CAA GGG CA-3’; GAPDH: sense 5’-CAA CTC ACT CAA GAT TGT CAG
Materials and Methods

Donor mice were tolerized with either 100 μg human PG and 2 mg DDA in 200 μl PBS at day 0 and day 21. Prior to the first immunization, donor mice were tolerized with either 100 μg human PG in 10 μl PBS intranasally (Nasal) or 1 mg PG orally (Oral) at days −6, −3, and −1. Control mice received OVA mucosally (control). Acceptor mice received 1 × 10^6 CD4^+ splenocytes from donor mice at day 20 isolated as described in Materials and Methods *+, p < 0.05 compared to control; **, p < 0.01 compared to control.

Results

**Mucosal Ag application prevents PGIA**

To explore whether mucosal tolerance suppressed disease in the chronic PGIA model, PG was applied on days −7, −5, and −3 either i.n. or intragastrically (i.g.). Control mice received OVA protein i.n. and/or i.g. All mice were subsequently immunized for the induction of arthritis on days 0 and 21.

Intranasal and oral application of PG before immunization significantly reduced the development of arthritis, as shown by the lower mean arthritis score when compared with control OVA-treated mice (Fig. 1). Also, the maximum arthritis score of the individual animals was reduced. OVA-treated controls showed an average maximum score of 7.4 ± 4.0, while tolerized animals after nasal treatment only reached a maximum score of 3.2 ± 2.6 and orally tolerized animals had a score of 2.7 ± 2.4 (Table I). Assessment of the mean day of arthritis onset revealed that mucosally treated mice not only developed less severe disease, but also that initial signs of disease occurred significantly later (Table I).

These data show that mucosal tolerance is a powerful means to reduce arthritis induction in the PGIA model.

**Mucosal Ag enhances Foxp3, IL-10, and TGF-β expression by CD4^+ splenocytes**

Subsequently, we analyzed the effect of mucosally applied Ag on differentiation of CD4^+ T cells within the paw DLNs and spleens of mucosally tolerized mice as based on cytokine profile and surface marker expression. DLN and spleen cells were isolated on day 40 after the first immunization, and single-cell suspensions were cultured for 24 h in the presence of 50 μg/ml PG. The last 4 h of culture brefeldin A was added to analyze the intracellular cytokine content. For expression of surface markers, cells were analyzed without prior in vitro reactivation.

Both intranasally and orally tolerized mice showed enhanced numbers of PG-specific cells producing IL-10 within the draining lymph nodes compared with OVA-treated controls. Fewer IL-10-producing cells in the OVA-treated control mice coincided with enhancement of the IFN-γ-producing cell population in the DLNs (Fig. 2).

Several studies have demonstrated that oral Ag application induced Tr cells residing within the CD4^+ spleen population. To address whether CD4^+ spleen cells obtained a regulatory phenotype in PGIA, cytokine expression within this population was assessed.

Within the spleen cell population of the protected mice, only few cells were found to produce IL-10 in an Ag-specific manner. Therefore, no differences as found in DLNs can be observed by flow cytometry. However, when CD4^+ cells were isolated to assess in situ expression of IL-10, TGF-β, and Foxp3 (Fig. 3), in both nasally and orally tolerized mice the levels of IL-10, TGF-β, and Foxp3 mRNA were enhanced in the splenic CD4^+ population compared with control-treated arthritic mice.

The observed differences in cytokine expression did not correlate with differences in surface marker expression of CD25,
CD103, or CD27 within the CD4⁺ population as measured by flow cytometry in both spleen and DLN-derived cells (data not shown).

Taken together, our data showed that PG-specific T cells in the DLNs of tolerized mice displayed a regulatory cytokine profile upon Ag recognition. Additionally, splenocytes of these mice showed enhanced transcription of IL-10, Foxp3, and TGF-β, all coding for proteins associated with immunomodulatory Tr cells.

Mucosal Ag induces CD4⁺ Tr cells in the spleen with the capacity to suppress arthritis

To determine whether the enhanced expression of regulatory molecules observed in spleen reflected functional differentiation of mucosal Tr cells, we isolated CD4⁺ splenocytes from intranasally tolerized, orally tolerized, and OVA-treated control mice. These CD4⁺ splenocytes, potentially containing Tr cells, were subsequently transferred i.v. to mice on day 20, before the second PG immunization on day 21.

As shown in Fig. 4, mice that received CD4⁺ splenocytes from nasally or orally tolerized mice only developed mild arthritis compared with control groups, indicating that CD4⁺ spleen cells from mucosally tolerized mice had obtained regulatory capacity. On the other hand, CD4⁺ splenocytes from OVA-tolerized control mice slightly enhanced the onset of arthritis, as several mice already showed signs of arthritis on day 26 whereas donor mice developed the first clinical arthritic scores on average on day 29 (Table I).

Next to a significant reduction of the clinical arthritis course, the maximum arthritis score was reduced when mice received 1 × 10⁶ CD4⁺ splenocytes from orally or intranasally treated animals (Table I).

Thus, oral or nasal application of PG before induction of arthritis seemed to have induced functional Tr cells that suppressed arthritis in already immunized acceptor mice.

Transferred mucosal Tr cells suppress both T and B cell immunity in arthritis

Because PG-specific autoantibodies are essential for inducing severe arthritis in PGIA, the effect of Tr cell transfer on the B cell response was studied. Both nasally and orally induced Tr cells suppressed the B cell-dependent Ab response as measured by the levels of Ag-specific IgG1 and IgG2a (Fig. 5).

Transfer of either orally or nasally induced Tr cells significantly reduced the Th1-mediated PG-specific IgG2a Ab levels in serum. Not only was the IgG2a response against immunizing human PG reduced, but the mouse PG-specific IgG2a response was significantly reduced upon transfer of Tr cells. In contrast, PG-specific IgG1 Ab levels were only significantly reduced by transfer of orally induced Tr cells. In summary, transfer of both nasally and orally induced Tr cells suppressed human and mouse PG-specific B cell responses.

FIGURE 4. CD4⁺ splenocytes transfer tolerance to arthritogenic immunization in recipient mice. All mice were immunized with 400 μg human PG and 2 mg DDA in 200 μl PBS at day 0 and day 21. Before the second immunization, mice received 1 × 10⁶ CD4⁺ splenocytes from mice that were tolerized as described in Fig. 1. Arthritis scores were analyzed three times a week (with a maximum score of 16 per mouse). Mean arthritis scores per group per day ± SD are shown. Data are the mean values of at least 5 mice per group ± SD. Data are representative of three separate experiments. *, p < 0.05 compared to control.

FIGURE 5. Reduced Ab response to human PG and murine PG after transfer of Tr cells. Mice were treated as described in Fig. 4, and at day 40 blood was drawn and serum was analyzed by ELISA for IgG1 and IgG2a to human and murine PG. Data are the mean values of at least 5 mice per group ± SD. Data are representative of three separate experiments. *, p < 0.05 compared to control.
To follow the effect of Tr cell transfer on infiltration of T cells and neutrophils into the joints of arthritic Tr cell recipients, we analyzed CD4 and MPO contents in joint infiltrates. Q-PCR analysis of cells isolated from the joints of Tr cell recipients showed a clear reduction of infiltrating CD4⁺ and MPO⁺ cells, indicating reduced influx of both CD4⁺ T cells and neutrophils (Fig. 6). This observation correlated with the reduced arthritis scores as shown in Fig. 4.

Tr cell acceptor mice show enhanced numbers of IL-10-producing T cells within the joint DLNs

To analyze the immunomodulatory role of the transferred Tr cells in recipient mice, we analyzed the proteoglycan-specific cytokine response in the joint DLNs. Single-cell suspensions of the paw DLNs were restimulated at 5×10⁶ cells/ml with 50 μg/ml PG in vitro during 18 h, and brefeldin A was added for the last 6 h. For the analysis of cytokine secretion in the culture supernatant, cells were stimulated for 72 h in the absence of brefeldin A.

Transfer of nasally and orally induced Tr cells enhanced not only the percentage of IL-10-producing CD4⁺ T cells (Fig. 7A), but also significantly enhanced the concentration of IL-10 in response to PG in the culture supernatant (Fig. 7C). Additionally, PG-specific IFN-γ secretion was significantly reduced in Tr cell recipients when compared with mice receiving control cells (Fig. 7B). In contrast, no significant differences were detected in the concentration of TNF-α (Fig. 7D) or the relative expression of IL-17 mRNA (Fig. 7G). Also, spleen cells from Tr cell recipients restimulated with PG in vitro produced significantly more IL-10 than did cells from control animals (data not shown).

Paw DLN cells showed significantly enhanced expression of IL-10 mRNA in situ in mice that received intranasally induced Tr cells compared with mice receiving control cells from controls (Fig. 7E). Also recipients of orally induced Tr cells showed enhanced relative expression of IL-10, although this effect was not statistically significant. Additionally, the Foxp3 expression in the DLNs of mice that received orally induced Tr cells was enhanced (Fig. 7F).

These data indicate that transfer of Tr cells from mucosally tolerized mice modulated the cytokine profile in PG-specific T cells in the paw DLNs as shown by the reduced IFN-γ production.

Tr cells proliferate in the DLNs and inhibit proliferation of naive T cells

Because it is unclear at present where transferred Tr cells exert their regulatory function, we sought to identify the location of Tr cell activation. Therefore, we labeled 5×10⁶ CD4⁺ mucosally induced Tr cells with CFSE before transfer into already immunized recipient mice. Subsequently, mice were immunized the next day and spleen and paw DLNs were isolated 4 days after the second PG immunization and were characterized for proliferation by flow cytometry of CFSE dilution.
Compared to oral tolerization, the induction of oral PG-specific Tr cells was more efficient, since the induction dose was 175-fold lower than that needed for the induction of nasally induced Tr cells (34–36). However, our study is the first showing that nasal application of PG was sufficient to induce functional Tr cells that are suppressive upon transfer without further treatment. This is in contrast with an earlier study in the PGIA model (33), which showed that continuous nasal treatment was needed to maintain tolerance after the induction of nasally tolerized Tr cells was 175-fold lower than that needed for the induction of oral Tr cells (34–36).

Our data confirm earlier findings on nasal tolerance in arthritis (37–39). However, our study is the first showing that nasal application of PG was sufficient to induce functional Tr cells that are suppressive upon transfer without further treatment. This is in contrast with an earlier study in the PGIA model (33), which showed that continuous nasal treatment was needed to maintain tolerance after transfer of splenocytes of tolerized mice to SCID acceptor mice. This difference can be explained by a difference in Tr cell population, because we transferred CD4$^+$ T cells to immune competent hosts instead of splenocytes to SCID mice. In the earlier study, transfer of unfractionated spleen populations may have led to cotransfer of potentially arthritogenic cells that contributed to disease induction.

Mucosal tolerance to Ags has been considered an effective means to prevent T cell-mediated immune responses to the same Ag. In humans, however, oral tolerance failed to suppress Ag-specific B cell responses to an exogenous Ag (12). B cells are known to play a crucial role in the pathogenesis of RA via the induction of autoantibodies, activation of autoreactive T cells, and formation of tertiary lymphoid structures (40). Modulation of the B cell response via mucosal tolerance induction could therefore strongly enhance therapeutic benefit. Herein we show that mucosal Tr cells are capable of suppressing the Th1-mediated Ag-specific IgG2a response irrespective of the site of their induction to both the tolerizing Ag and the murine PG. However, only orally induced Tr cells also suppress the Ag-specific IgG1 response, indicating a more general suppression of both Th1 and Th2 cell-mediated immune responses. This is in agreement with an earlier study showing that orally tolerized T cells can no longer provide cognate help to B cells (41). Given our finding that nasal tolerance was effectively suppressing disease, we may conclude that suppression of IgG1 is not essential for suppression of disease. These findings indicate that although both oral and nasal tolerance can induce tolerance via the induction of Tr cells, the suppressive mechanisms might differ.

Even though both oral and nasal Ag application resulted in suppression of disease, no obvious changes in cell surface marker expression of T cells in DLNs or the spleen of treated mice were detected. This is in agreement with recent studies exploring the
phenotype of mucosally induced Tr cells rapidly after Ag application, as these studies showed that such Tr cells can hardly be distinguished from other recently activated T cells and that regulatory capacity resides in both CD25+ and the CD25+ populations (34–36). Additionally, phenotypic differences in a small population will not be reflected by differences in the entire CD4+ T cell population in spleen. However, the observation that differences in regulatory markers are present in spleen mRNA in combination with their ability to transfer tolerance to immunized recipients suggests that these Tr cells do reside in this tissue.

In this study we collected evidence that mucosally induced Tr cells were not only able to migrate into the joint DLN, but also that this reduced proliferation compared with Tr cells is due to the activation that occurred in the severely diseased donor mice. The data indicate that mucosal Tr cells suppressed arthritis development by suppressing proliferation of pathogenic T cells in the joint DLNs, thereby reducing subsequent immune responses at both the T cell and B cell levels, in line with the findings of enhanced IL-10 expression in CD4+ T cells and reduced IgG2a levels. Herein we show that both oral and nasal Ag application can induce functional Tr cells in the chronic and relapsing PGIA model. It is possible that effective translation into therapeutic application in humans will lead to combination therapy with other antiinflammatory approaches, such as anti-TNF-α. Such combination of therapies will then broadly target autoaggressive T effector cells while inducing or expanding Ag-specific Tr cells, diverting the autoimmune response into a more regulatory type (42). Recently, the effectiveness of such a combined approach was demonstrated in RA patients (43, 44). The skewing of Ag-specific inflammatory responses toward more tolerogenic responses can become a major addition to available therapeutic options for autoimmune diseases.

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Disclosures
The authors have no financial conflicts of interest.

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