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Mucosal Anti-CD3 Monoclonal Antibody Attenuates Collagen-Induced Arthritis That Is Associated with Induction of LAP⁺ Regulatory T Cells and Is Enhanced by Administration of an Emulsome-Based Th2-Skewing Adjuvant

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Mucosal Anti-CD3 Monoclonal Antibody Attenuates Collagen-Induced Arthritis That Is Associated with Induction of LAP⁺ Regulatory T Cells and Is Enhanced by Administration of an Emulsome-Based Th2-Skewing Adjuvant

Henry Yim Wu, Ruth Maron, Ann-Marcia Tukpah, and Howard L. Weiner

Mucosal (nasal or oral) administration of anti-CD3 mAb is effective in ameliorating animal models of autoimmunity (experimental autoimmune encephalomyelitis, diabetes, and lupus) by inducing LAP⁺ regulatory T cells. We tested this approach in an arthritis model using type II collagen. We found that nasal anti-CD3 was more effective than oral anti-CD3 in attenuating the development of arthritis. Nasal anti-CD3 induced a LAP⁺ regulatory T cell that secreted high levels of IL-10 and suppressed collagen-specific T cell proliferation and anti-collagen Ab production. However, neither nasal nor oral anti-CD3 attenuated disease when given to animals with ongoing arthritis, and this was associated with a lack of induction of LAP⁺ regulatory T cells. We found, however, that coadministration of a novel emulsome adjuvant, which enhances Th2 responses, resulted in the induction of LAP⁺ regulatory T cells and suppression of ongoing arthritis by both nasal and oral anti-CD3. Suppression of arthritis by mucosal anti-CD3 was associated with less joint damage, a decrease of TNF-α and IFN-γ mRNA expression in joints, and a reduction in anti-collagen Abs. These results demonstrate that mucosal anti-CD3 therapy may serve as a therapeutic approach in arthritis and that the biologic effect is enhanced by an emulsome-based adjuvant.
in CFA (Sigma-Aldrich, St. Louis, MO) divided equally in five intradermal injection sites. Three weeks after the primary CII immunization, mice received a booster injection of 100 μg soluble CII i.p. We began observing the mice 7 d after the booster immunization. In therapeutic studies, we induced arthritis by CII immunization. Immediately after the booster immunization, mice received nasal or oral IC or anti-CD3 emulsified in 5% emulsol as described above. Mice were then followed for 100 d. To assess arthritis, mice were scored on a scale of 0–4: 0, no signs of disease; 1, erythema and mild swelling of the tarsus; 2, moderate erythema and swelling of the tarsus and ankles; 3, severe swelling of the tarsus and ankles; and 4, ankylosis and bone deformity. The arthritic score for each mouse was the combined score of all four paws.

**T cell proliferation**

Cervical (superficial and deep), mesenteric lymph node cells or splenocytes were cultured in triplicate at 0.5 × 10^6/well in the presence of 100 μg CII in 96-well round bottom microtiter plates (Corning Glass, Corning, NY) for 72 h at 37°C with 5% CO2 in a humidified incubator. Tissue culture medium was RPMI 1640 supplemented with 1% L-glutamine, 1% penicillin/streptomycin (BioWhittaker, Walkersville, MD), and 10% FCS. Cultures were pulsed with 1 μCi tritiated thymidine (PerkinElmer, Wellesley, MA) for the last 12 h. Tritiated thymidine incorporation was measured using a liquid scintillation beta counter (Wallac; PerkinElmer). Cell proliferation was expressed in Δ CPM.

**Histology**

Mouse paws were fixed in 10% formalin (Fisher Scientific, Pittsburgh, PA). Before H&E staining, paws were embedded in paraffin (Tissue-Tek, Torrance, CA), and 10 μM sections were cut on a cryostat. Slides were examined in a blinded fashion.

**Cytokine detection**

The level of cytokines produced in vitro by cell cultures was determined using BD OptEIA ELISA set and reagent set B (BD Biosciences). Samples were tested in duplicate using manufacturer’s recommended assay procedures. For the detection of cytokines, cell culture supernatants were harvested at different time points (40 h for IL-2, IL-4, IL-6, IL-10, and IFN-γ or 72 h for TGF-β). The level of cytokines produced in vitro by cell cultures was determined using BD OptEIA ELISA set and reagent set B (BD Biosciences). Samples were tested in duplicate using manufacturer’s recommended assay procedures. For the detection of cytokines, cell culture supernatants were harvested at different time points (40 h for IL-2, IL-4, IL-6, IL-10, and IFN-γ or 72 h for TGF-β).

**Flow cytometry**

Cells were washed with PBS containing 1% BSA (PBS/BSA; BioWhittaker). FcγRs were blocked by incubation with anti-mouse CD16/CD32 Ab (BD Biosciences). Cells were washed twice before being stained with fluorescent-labeled anti-mouse Abs (1 μg/10^6 cells/well) or relevant IC Ab for 30 min at 4°C in dark. After staining, cells were washed again with PBS/BSA before FACS (FACScan; BD, Franklin Lakes, NJ). Intracellular staining was carried out as described previously (12). All FACS data were analyzed using FlowJo software (Tree Star, Ashland, OR).

**ELISA for serum anti-CII Abs**

Abs were measured as described previously (14). Briefly, chicken CII at 5 μg/ml in 0.1 M sodium bicarbonate (pH 9.2) was coated onto 96-well plates overnight at 4°C. Plates were washed with PBS and incubated for 2 h at room temperature with PBS containing 1% BSA, 0.5 μl/ml Tween 20, and 2.5% normal goat serum to neutralize nonspecific binding sites. Plates were washed with PBS, and serum samples in different dilutions were added (50 μl/well). For the detection of total IgG, IgG1, IgG2a, or IgG2b Abs, 50 μl/well HRP-conjugated rat anti-mouse Ab (BD Biosciences) at 0.001 μg/ml was added and incubated at room temperature for 1 h.

**Polymerase chain reaction**

Skin was removed from paws before being homogenized in TRIZol (Sigma-Aldrich). RNA was extracted from joint homogenate using RNAeasy columns (Qiagen, Valencia, CA). cDNA was transcribed as recommended (Applied Biosystems, Foster City, CA). RT-PCR was carried out using cDNA from joint homogenates. All primer/probe mixtures were obtained from Sigma-Genosys (The Woodlands, TX). PCR products were visualized on 1% agarose gel. TaqMan analysis was performed on AB 7500 Fast System (Applied Biosystems). Gene expression was normalized to the expression of β-actin. All primer/probe mixtures were obtained from Applied Biosystems.

**Statistical analysis**

Statistical differences in cell proliferation, cytokine production, and serum Ab levels were derived from Student t test. The Wilcoxon rank-sum test was used for all of the pairwise group comparisons. A p value <0.05 was considered significant.

**Results**

**Mucosal anti-CD3 prior to induction of CIA attenuates disease**

We administered 0.5 or 5 μg anti-CD3 nasally or orally, respectively, in accordance with doses used in our previous studies (11, 12). We used the F(ab′)2 fragment of anti-CD3 Ab as done in previous studies (12). DBA/1 mice received IC or anti-CD3 nasally (Fig. 1A) or orally (Fig. 1E) before immunization with CII. As
shown in Fig. 1A, nasal anti-CD3 significantly lowered the severity and incidence (Table I) of arthritis compared with nasal IC in mice. In addition, we found significantly lower mRNA expression of the proinflammatory cytokines IFN-γ (p = 0.002) and TNF-α (p = 0.0005) in joints of nasal anti-CD3–treated mice (Fig. 1B) and decreased levels of IgG and IgG subclass Abs to chicken CII in anti–CD3–treated animals (Fig. 1C). Oral anti-CD3 given before the induction of CIA showed a trend toward disease attenuation but did not achieve statistical significance (p = 0.06) (Fig. 1D) and less prominent changes in cytokine expression in joints (Fig. 1E) compared with nasal anti-CD3 (Fig. 1B). No changes in serum Ab levels were observed in oral anti–CD3–treated animals (data not shown).

**Nasal anti-CD3 suppresses Ag-specific T cell proliferation and induces IL-10 production**

We next examined the effect of nasal anti-CD3 on Ag-specific T cell proliferation and cytokine production. We found a significant reduction in T cell proliferation to CII both in cervical lymph node (p = 0.045) and spleen (p = 0.002) (Fig. 2A) demonstrating that T cell regulation occurred both locally and systemically following nasal anti-CD3 treatment. We did not observe apoptosis in CD4+ T cells from cervical lymph nodes or spleens (data not shown) as has been reported for i.v. anti-CD3 treatment (15). In addition, we observed a significant (p = 0.001) decrease in IL-2 production (Fig. 2B) and downregulation of both Th1 (IFN-γ) and Th2 (IL-4) type cytokine production (Fig. 2B), which correlates with our finding of reduced Th1- and Th2-linked IgG subclass Abs in serum of nasal anti–CD3–treated mice (Fig. 1D). In accordance with our previous findings (11, 14, 16), we found a high level of IL-10 production by T cells of nasal anti-CD3–treated mice (Fig. 2B, 2C). We did not observe changes in proliferation (Fig. 2D) or cytokine production (Fig. 2E) by splenocytes or mesenteric lymph node cells after oral anti-CD3 treatment.

**Nasal anti-CD3 induces LAP+ T cells**

We have previously shown that nasal or oral anti-CD3 induced a LAP+ regulatory T cell that suppresses experimental autoimmune encephalomyelitis (8), type I and II diabetes (9, 10), and lupus (11, 12) in mice. Thus, we investigated LAP expression following nasal anti-CD3 in DBA/1 mice. We found an upregulation of LAP expression in both CD4+CD25+ and CD4+CD25+ T cells in cervical lymph node of mice nasally treated with anti–CD3 (Fig. 3A). We carried out comparative studies of LAP expression in individual mice (eight per group) treated with nasal anti-CD3 and found that there was a significant upregulation of LAP+ T cells in both cervical lymph node and spleen (Fig. 3B, 3C). This correlates well with our findings of local and systemic downregulation of proliferative responses to CII (Fig. 2A), indicating that LAP+ T cells are associated with suppression of Ag-specific immune responses. Oral anti-CD3 did not upregulate LAP expression on T cells in mesenteric lymph nodes or spleen.

**Table I. Incidence of arthritis following nasal anti-CD3**

<table>
<thead>
<tr>
<th>Arthritis Type</th>
<th>Nasal IC</th>
<th>Nasal Anti-CD3</th>
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</thead>
<tbody>
<tr>
<td>Severe arthritis (&gt;3)</td>
<td>8</td>
<td>1*</td>
</tr>
<tr>
<td>Mild arthritis (&lt;2)</td>
<td>0</td>
<td>3*</td>
</tr>
<tr>
<td>No arthritis</td>
<td>0</td>
<td>4*</td>
</tr>
</tbody>
</table>

DBA/1 mice (eight per group) received three doses of 0.5 μg IC or anti-CD3 Ab nasally every other day before immunization with CII. Arthritis was scored as indicated in Materials and Methods. Incidence of arthritis at 100 d postinduction in each treatment group is shown. Data were analyzed by χ² test.

*p < 0.05 compared with IC-treated controls.

**Figures**

**FIGURE 2.** T cell hyporesponsiveness and cytokine production. A, DBA/1 mice (three per group) received three doses of 0.5 μg IC (open bars) or anti-CD3 (filled bars) Ab nasally every other day before immunization with CII. Seven days after CII immunization, cervical lymph node cells or splenocytes were cultured in vitro with CII (100 μg/ml). B, Splenocyte culture supernatants were used in ELISA. C, Cervical lymph node cells were surface stained with CD4 and LAP and intracellularly stained with IL-10 Abs. IL-10 in gated CD4+LAP+ T cells is shown in histogram. D, DBA/1 mice (three per group) received five consecutive doses of 5 μg IC or anti-CD3 Ab orally before immunization with CII. Seven days after CII immunization, mesenteric lymph node cells or splenocytes were cultured in vitro with CII (100 μg/ml). E, Splenocyte culture supernatants were used in ELISA. These experiments were repeated once with similar results.
As shown in Fig. 2C that nasal anti-CD3 generated IL-10–producing CD4+LAP+ T cells, we investigated a role of IL-10 in local immune response in target tissue. We examined IL-10 mRNA expression in joints of mice 100 d postnasal treatment. Fig. 3F shows that nasal anti-CD3 but not IC led a significant increase in IL-10 mRNA expression. This suggests that nasal anti-CD3 attenuated CIA development that is associated with an induction of IL-10–producing LAP+ regulatory T cells.

Both nasal and oral anti-CD3 suppresses ongoing arthritis when given together with emulsome

To test the therapeutic effect of mucosal anti-CD3, we treated animals with ongoing arthritis after disease induction with nasal or oral anti-CD3. We found no effect on the severity of arthritis or immune responses including serum anti-collagen Abs or induction of LAP+ regulatory T cells with either mucosal route (data not shown). These results suggest that nasal anti-CD3 attenuated CIA development that is associated with an induction of IL-10–producing LAP+ regulatory T cells.

(Fig. 3D, 3E). As shown in Fig. 2C that nasal anti-CD3 generated IL-10–producing CD4+LAP+ T cells, we investigated a role of IL-10 in local immune response in target tissue. We examined IL-10 mRNA expression in joints of mice 100 d postnasal treatment. Fig. 3F shows that nasal anti-CD3 but not IC led a significant increase in IL-10 mRNA expression. This suggests that nasal anti-CD3 attenuated CIA development that is associated with an induction of IL-10–producing LAP+ regulatory T cells.

We next measured the effect of emulsome given together with mucosal anti-CD3 on collagen Abs, joint destruction, and the induction of LAP+ T cells. We found that both nasal and oral anti-CD3 given together with emulsome decreased anti-CII Abs when measured at the end of the experiment (100 d after disease induction; Fig. 4C).

Joint destruction characterized by inflammatory hyperplasia, muscle fiber thinning, and bone erosion was prevented by nasal or oral anti-CD3 plus emulsome (Fig. 4D). This was associated with a downregulation of mRNA expression of IFN-γ and TNF-α in the joints (Fig. 4E). Emulsome alone given nasally or orally did not affect these parameters (data not shown). We hypothesized that the effect of emulsome was related to the induction of LAP+ regulatory T cells by mucosal anti-CD3. To test this, we examined LAP expression on CD4+CD25+T cells in mesenteric lymph node and spleen of mice at 100 d after mucosal anti-CD3 and emulsome combination therapy. We found that anti-CD3 plus emulsome given either nasally (Fig. 5A, 5B) or orally (Fig. 5C, 5D) significantly increased the percentage of LAP+CD4+CD25+ T cells compared with emulsome given together with IC. Thus, the therapeutic effect of mucosal anti-CD3 plus emulsome in arthritic mice is associated with the induction of LAP+ regulatory T cells.
Discussion

We show in this paper that nasal anti-CD3 attenuated the development of CIA in DBA/1 mice and that the therapeutic effect was associated with an induction of LAP⁺ regulatory T cells. Nasal anti-CD3 given prior to the induction of CIA attenuated the development of disease and downregulated anti-collagen Ab production as well as IFN-γ and TNF-α mRNA expression in joints. Previously, we also observed decreases in Ab production and cytokine secretion in lupus-prone mice treated with nasal anti-CD3 (11). This effect was mediated by IL-10–secreting LAP⁺ regulatory T cells that controlled the differentiation and expansion of follicular helper T cells leading to downregulation of B cell activation (11). In the current study, our finding of a significant decrease in collagen-specific T cell proliferation following nasal anti-CD3 suggests that a similar regulatory mechanism may exist in animal models of arthritis. In support of this view, we found that the effect of nasal anti-CD3 was accompanied by a significant increase in IL-10 production and LAP⁺ regulatory T cell differentiation.

In contrast, we did not find a significant effect of oral anti-CD3 in preventing CIA or inducing immunomodulatory effects, although we did observe a trend in reduction of disease severity. The reason for differences in nasal versus oral anti-CD3 in preventing arthritis is not clear. It could relate to dosing, because we did not test a wide range of doses given orally in the collagen model. It is also possible that tolerogenic mediators, which are functional in the upper airways, may be defective in the gut mucosa of DBA/1 mice. Consistent with this view, we have found increased apoptosis of CD11c⁺ dendritic cells in mesenteric lymph nodes of DBA/1 mice (H.Y. Wu, unpublished data).

Induction of tolerance prior to disease induction in animal models may not be applicable to clinical situations in which patients have already developed disease. Thus, we tested the therapeutic effect of mucosal anti-CD3 following CIA induction in DBA/1 mice. Contrary to the positive effects seen with anti-CD3 treatment before CIA induction, we observed no effect on disease or immune measures including serum anti-collagen Abs and the induction of LAP⁺ regulatory T cells when mice were treated with nasal or oral anti-CD3 after CIA induction. We hypothesized that the lack of effect in mice with ongoing disease may be due to the inhibitory effect of systemic inflammation on LAP⁺ regulatory
T cell generation by mucosal anti-CD3. A shift in immune response toward an inflammatory Th1 phenotype during the development of arthritis in mice (18) and in arthritic joints of patients with rheumatoid arthritis (19) has been reported.

To address this issue, we investigated the coadministration of emulsome, an adjuvant that biases toward Th2-type immune responses (17). Emulsome is a novel lipoidal particulate vehicle with features intermediate between liposomes and oil-in-water emulsions (17, 20). Emulsome has a bioadhesive polymer that confers mucoadhesive properties to the lipoidal particles to improve attachment to mucosal surfaces. Feeding of emulsome suppressed IFN-γ production by T cells following in vitro Ag stimulation (R. Maron, unpublished data). Therefore, we tested the effect of a combination therapy with mucosal anti-CD3 and emulsome in mice following CIA induction. We found that nasal or oral emulsome restored the suppressive effects of anti-CD3 and attenuated ongoing disease in mice. A combination of mucosal anti-CD3 and emulsome suppressed the production of anti-CII Abs and improved the severity of joint pathology by reducing inflammatory cytokines in joints. This was associated with an upregulation of LAP⁺ T cells indicating that, indeed, induction of LAP⁺ regulatory T cells by mucosal anti-CD3 was associated with suppression of disease processes. Therefore, dampening inflammatory responses by emulsome promotes the generation of LAP⁺ regulatory T cells by mucosal anti-CD3. To our knowledge, this is the first study that shows a combination of anti-CD3 and adjuvant is efficacious in controlling ongoing arthritis in mice. This puts forward the possibility of a combinational therapy in other autoimmune diseases where therapeutic efficacy is hindered by systemic inflammation during active disease. In this regard, we have found that administration of β-glucosylceramide enhances the effect of oral anti-CD3 in a model of type II diabetes (10).

Recent studies have demonstrated that systemic injection of anti-CD3 in DBA/1 mice suppressed CIA by expanding CD4⁺/CD8⁺ CD25⁺Foxp3⁺ regulatory T cells (21). This may be due to an initial deletion of pathogenic T cells, thus allowing the expansion of Foxp3⁺ regulatory T cells (22). In addition, CD8⁺CD25⁺Foxp3⁺ regulatory T cells have been induced after i.v. anti-CD3 in humans (23). We have recently characterized human LAP⁺ regulatory T cells (24). In contrast to systemic delivery, mucosal administration of anti-CD3 induced CD4⁺LAP⁺ regulatory T cells without significant upregulation of Foxp3 expression (8, 11, 12). Furthermore, we observed neither deletion of T cells nor downregulation of CD3 expression following mucosal anti-CD3. Thus, different mechanisms are involved in the induction of regulatory T cell by mucosal verses systemic delivery of anti-CD3 (25).

We previously demonstrated that nasal or oral administration of CII in DBA/1 mice before induction of CIA suppressed IFN-γ production by CII-specific pathogenic T cell as well as TNF-α and IL-6 mRNA expression in joints of orally tolerized mice (26).

Table II. Incidence of arthritis following mucosal anti-CD3 given with emulsome

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Severe arthritis (clinical score &gt;3)</th>
<th>Mild arthritis (clinical score &lt;2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nasal EMU</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Nasal IC + EMU</td>
<td>2*</td>
<td>6*</td>
</tr>
<tr>
<td>Nasal Anti-CD3 + EMU</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Oral EMU</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oral IC + EMU</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oral Anti-CD3 + EMU</td>
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* p < 0.05 compared with IC-treated controls.

FIGURE 5. Induction of LAP⁺ regulatory T cells following mucosal anti-CD3 and emulsome. DBA/1 mice (eight per group) were nasally treated with 0.5 µg or fed 5 µg IC or anti-CD3 and emulsome after induction of CIA. Mice were followed for 100 d, and at which point splenocytes were stained with 7AAD, anti-mouse CD4, CD25, and LAP Abs. 7AAD⁺CD4⁺ live T cells were gated, and percentages of CD4⁺CD25⁺LAP⁺ or CD4⁺CD25⁻LAP⁺ T cells in spleen of individual mice are shown. A and B represent mice treated nasally. C and D represent mice treated orally. Each symbol represents one mouse. Mean values are represented by cross bars. These experiments were repeated once with similar results.
Disease suppression was associated with a shift in immune responses from a Th1 to a Th2 phenotype. Interestingly, immune deviation from Th1 to Th2 responses was also observed in previous nasal tolerance studies using histone peptides in animal models of lupus (14). More recently, Broere et al. (27) demonstrated that oral or nasal administration of proteoglycan induced functional IL-10–producing CD4+ regulatory T 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 (27). In this study, we show that mucosal anti-CD3 not only attenuated the development of CIA when given in a preventive regimen but also arrested ongoing disease. Mucosal anti-CD3 not only attenuated the development of arthritis severity and incidence (27). In this study, we show that mucosal anti-CD3 not only attenuated the development of CIA when given in a preventive regimen but also arrested ongoing disease. In this study, we show that mucosal anti-CD3 not only attenuated the development of CIA when given in a preventive regimen but also arrested ongoing disease.

In summary, we have shown that mucosal anti-CD3 Ab attenuates the development of CIA in DBA/1 mice. Nasal anti-CD3 suppresses the development of CIA in lupus prone mice attenuated arthritis severity and incidence (27). In this study, we show that mucosal anti-CD3 not only attenuated the development of CIA when given in a preventive regimen but also arrested ongoing disease.

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Disclosures
H.L.W. is a consultant for Nasvax. The other authors have no financial conflicts of interest.

References