Immune Modulatory Treatment of Trinitrobenzene Sulfonic Acid Colitis with Calcitriol Is Associated with a Change of a T Helper (Th) 1/Th17 to a Th2 and Regulatory T Cell Profile

Carolin Daniel, Nico A. Sartory, Nadine Zahn, Heinfried H. Radeke, and Jürgen M. Stein

First Department of Internal Medicine Zentrums fuer Arzneimittelforschung, Entwicklung und Sicherheit (ZAFES) (C.D., N.A.S., N.Z., J.M.S.), Pharmazentrum Frankfurt ZAFES (C.D., H.H.R.), Johann Wolfgang Goethe University of Frankfurt am Main, Frankfurt, Germany

Received June 13, 2007; accepted September 28, 2007

ABSTRACT

A number of recent studies testify that calcitriol alone or in combination with corticosteroids exerts strong immune modulatory activity. As a new approach, we evaluated the protolerogenic potential of calcitriol and dexamethasone in acute Th1 helper (Th1)-mediated colitis in mice. A rectal enema of trinitrobenzene sulfonic acid (TNBS) (100 mg/kg) was applied to BALB/c mice. Calcitriol and/or dexamethasone were administered i.p. from days 0 to 3 or 3 to 5 following the instillation of the haptenating agent. Assessment of colitis severity was performed daily. Colon tissue was analyzed macroscopically and microscopically, and myeloperoxidase activity, as well as cytokine levels [tumor necrosis factor-α, interferon-γ, interleukin (IL)-12p70, IL-1β, IL-10, IL-4] were determined by enzyme-linked immunosorbent assay, T-bet, GATA family of transcription factors, a Th2 master regulator (GATA3), Foxp3, cytotoxic T-lymphocyte-associated antigen 4 (CTLA4), IL-23p19 and IL-17 expression by immunoblot analysis. The combination of the steroids most effectively reduced the clinical and histopathologic severity of TNBS colitis. Th1-related parameters were down-regulated, whereas Th2 markers like IL-4 and GATA3 were up-regulated. Apart from known steroid effects, calcitriol in particular promoted regulatory T cell profiles as indicated by a marked increase of IL-10, TGFβ, FoxP3, and CTLA4. Furthermore, analysis of dendritic cell mediators responsible for a proinflammatory differentiation of T cells revealed a significant reduction of IL-12p70 and IL23p19 as well as IL-6 and IL-17. Thus, our data support a rationale for a steroid-sparing, clinical application of calcitriol derivatives in inflammatory bowel disease. Furthermore they suggest that early markers of inflammatory dendritic cell and Th17 differentiation qualify as new target molecules for both calcitriol and highly selective immune-modulating vitamin D analogs.

Calcitriol has been identified in a number of studies as a prominent negative regulator of Th1-type immune responses, whereas Th2 responses are not affected or even augmented as indicated by an induction of T1/ST2 (Schmitz et al., 2005; Wang et al., 2005). Although these effects have been preferentially explained by direct effects on lymphocytes, subsequent studies clearly supported a role of calcitriol in modulating monocyte differentiation or dendritic cell (DC) maturation (Griffin et al., 2003). Calcitriol clearly reduced the transition of the innate immune stimulation to an adaptive inflammatory immune response by interfering with signal transducers and activators of transcription, interferon regulatory factor 1/4, and possibly with the molecular elements involved in cross-presentation (Penna and Adorini, 2000; Muthian et al., 2006). With respect to myeloid DC development, the phenotype of calcitriol-conditioned DC reflects an immature status with poor capacity to induce antigen-specific T cell proliferation and a tendency to promote tolerance in vivo (Mellman and Steinman, 2001).

The immunoregulatory properties of calcitriol have been demonstrated in different models of autoimmune diseases (systemic lupus erythematosus, allograft rejection, autoimmune diabetes mellitus, and experimental allergic encephalomyelitis), in which a substantial amelioration was observed following treatment with calcitriol. These data from animal
models, either explainable by a skewing effect of calcitriol on the differentiation of DC or a direct activity in lymphocytes, support epidemiologic studies in humans indicating that vitamin D receptor (VDR) genotype and vitamin D status are definite risk factors for autoimmune diseases (Cantorna et al., 2004).

Glucocorticoids (GCs) are effectively applied for the treatment of the general inflammatory sequelae of both Th1- and Th2-mediated disorders, including rheumatoid arthritis and asthma (Wilckens and De, 1997; Karin, 1998). They inhibit both T cells and antigen-presenting cells at the level of proliferation and cytokine production (Blotta et al., 1997). In addition, it was shown that in vitro, a combination of dexamethasone and calcitriol induced high numbers of IL-10-producing T cells (Barrat et al., 2002).

Recent studies of inflammatory bowel diseases (IBDs) encompassing Crohn’s disease (CD) and ulcerative colitis suggest that these diseases are attributed to inappropriate and/or excessive responses to antigens present in the normal bacterial microflora. Th1/Th2 cytokine profiles characterize CD as a preferentially Th1 cell-mediated disease initiated by DC-derived IL-12 and/or IL-23 (Uhlig et al., 2006). Although it is not clear which of these DC factors dominates in local CD pathophysiology, recently, Th17 cells have been defined to comprise an IL-23-induced Th cell lineage with a proinflammatory role in autoimmunity and tissue inflammation (Betteli et al., 2006, 2007). As a model of hapten-induced colitis in mice caused by rectal instillation of trinitrobenzene sulfonic acid (TNBS)-colitis exhibits features comparable with such a Th1 autoimmune process. Consequently, it was demonstrated that agents that block a Th1 response or promote a Th2 profile are reducing disease symptoms and progression (Neurath et al., 1995). Herein, we describe an in vivo setting the inhibitory potential of calcitriol on Th1 and for the first time on Th17 (IL-17-producing T helper cells) effector functions in corticosteroid-treated mice with TNBS colitis.

Materials and Methods

Mouse Experiments

Male, 8-week old BALB/c mice weighing approximately 20 g were obtained from Charles River Laboratories (Sulzfeld, Germany). All studies were performed under approval of the Ethics Committee of Darmstadt/Hessen (Germany, F133/03) and are in agreement with the guidelines for the proper use of animals in biomedical research. The mice were kept in polycarbonate cages in temperature-controlled rooms with a 12-h light/dark cycle and fed standard mouse chow and tap water. At the end of the experiments, mice were sacrificed by cervical dislocation under isoflurane anesthesia; and 2) established colitis, dexamethasone and/or calcitriol were administered i.p. 2 h before the instillation of the TNBS enema and the after 3 days. On day 3 the colon was removed following cervical dislocation under isoflurane anesthesia; and 2) established colitis, dexamethasone and/or calcitriol were administered from days 3 to 5 following the instillation of the TNBS enema. On day 5, the colon was removed.

Induction of Colitis by the Haptenating Agent TNBS

The haptenating agent TNBS (2,4,6-TNBS; Sigma-Aldrich, Deisenhofen, Germany) was used at a concentration of 2% in 45% ethanol. For induction of colitis TNBS was administered [100 mg/kg body weight (BW)] to slightly anesthetized mice through a 3.5-F catheter carefully inserted into the rectum. The catheter tip was inserted 4 cm proximal to the anal verge. To ensure distribution of the TNBS within the entire colon and cecum, mice were held in a vertical position for 1 min after the instillation of the TNBS enema. Control animals were administered 45% ethanol alone using the same technique.

Administration of Calcitriol and/or Dexamethasone and Study Design

Calcitriol was purchased from Biomol (Hamburg, Germany), dissolved in ethanol at a concentration of 1 × 10−2 M and kept at −80°C until use. Dexamethasone (D-2915, water-soluble; Sigma-Aldrich) was dissolved in water to prepare a stock solution at a concentration of 6 mg/ml and stored at 4°C. Calcitriol was administered i.p. at a dose of 0.2 μg/kg BW in 0.9% NaCl solution, containing 0.085% Myrj53 (Sigma, Deisenhofen, Germany), the solutions were prepared daily. Dexamethasone was used at doses from 0.6 to 1.2 mg/kg, respectively. Two protocols were used. 1) Acute ongoing colitis, dexamethasone and/or calcitriol were administered i.p. 2 h before the instillation of the TNBS enema and the after 3 days. On day 3 the colon was removed following cervical dislocation under isoflurane anesthesia; and 2) established colitis, dexamethasone and/or calcitriol were administered from days 3 to 5 following the instillation of the TNBS enema. On day 5, the colon was removed.

Analytical Procedure for Determining Serum Calcium and Creatinine

Serum calcium levels were determined after treatment at the end of experiment. The concentration was measured by the calcium-cresolphthalein colorimetric assay according to the manufacturer’s instructions (Hitado, Möhnesee Delecke, Germany). Serum creatinine levels were measured using the alkaline picrate method (Hitado).

Assessment of Inflammation and Colitis Severity

Clinical Activity Score of Colitis. For the assessment of the clinical severity of colitis the BW, as well as the stool consistency and rectal bleeding, were examined daily. The clinical activity score of colitis was determined independently by two investigators being unaware of the treatment protocol using a scoring system described previously in detail (Hartmann et al., 2000). In brief, the loss of BW was scored as follows: 0, no weight loss; 1, weight loss of 1 to 5%; 2, weight loss of 5 to 10%; 3, loss of 10 to 20%; and 4, weight loss > 20%. The assessment of diarrea (stool consistency) was scored as follows: 0, normally formed pellets; 2, pasty and semiformal pellets; and 4, liquid stools. Bleeding was scored as follows: 0, no blood in hemocult; 2, positive hemocult; and 4, gross bleeding from the rectum. The results of these scoring parameters were added resulting in a total clinical score ranging from 0 (healthy) to 12 (maximal ill/activity of colitis). The length and weight of the colon were used as indirect markers of disease-associated intestinal wall thickening correlating with the intensity of inflammation.

Macroscopic Scoring System. The assessment of the macroscopic colonic damage was performed using the scoring system of Wallace and Keenan (1990), taking into account the area of inflammation and the presence and absence of ulcers. The criteria for the evaluation of macroscopic damage were based on a semiquantitative scoring system. Features were graded as follows: 0, no ulcer, no inflammation; 1, no ulcer, local hyperemia; 2, ulceration without hyperemia; 3, ulceration and inflammation at one site only; 4, two or more sites of ulceration and inflammation; and 5, ulceration extending more than 2 cm.

Histological Analysis of the Colon. For histological examination, a sample of colonic tissue located precisely 3 cm above the anal canal was obtained from the mice of all treatment groups. The colonic tissues were fixed in 10% neutral buffered formalin and embedded in paraffin for histological analysis. Four-micrometer-sections were deparaffinized with xylene and stained with hematoxylin and eosin using routine techniques. Tissues were graded semiquantitatively from 0 to 5 in a blinded fashion according to previously described criteria (Boirivant et al., 1998).

Measurement of Myeloperoxidase Activity. The MPO activity assay was performed using a modification of the method described by Bradley et al. (1982). The enzyme activity was determined photometrically as the MPO-catalyzed change in absorbance occurring in
the redox reaction of 3,3,5,5-tetramethylbenzidine dihydrochloride (TMB-substrate; Sigma-Aldrich) at 650 nm. MPO (Sigma-Aldrich) was visualized in ice-cold complete lysis buffer. The homogenates were then centrifuged at 4°C for 20 min. Aliquots of the resulting extracts were analyzed for their protein content using the Bio-Rad colorimetric assay according to the Bradford method (Bio-Rad Laboratories, Muenchen, Germany) and stored at -80°C until use.

### Cytokine Assays

The amount of murine TNFα, interferon-γ, IL-12p70, IL-6, IL-10, TGFβ, IL-1β, and IL-4 in the colonic protein lysates were quantified by commercially available enzyme-linked immunosorbent assay kits (R&D Systems, Abingdon, UK) according to the manufacturer’s instructions and adapted to the protein content of the colonic tissue sample.

### Western Blot Analysis

After addition of sample buffer to the colonic protein extracts and boiling samples at 95°C for 5 min, 150 μg of total protein lysate was separated on a 10% SDS-polyacrylamide gel (T-bet, GATA3, FoxP3, TNBS-treated mice were treated i.p. with dexamethasone and/or calcitriol from days 0 to 3 following the TNBS enema. Serum calcium and creatinine were determined following a 3-day treatment regimen. Data represent mean ± S.E.M. from three separate experiments (eight mice per group per experiment).

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>Serum Calcium</th>
<th>Serum Creatinine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.91 ± 0.32</td>
<td>0.95 ± 0.02</td>
</tr>
<tr>
<td>TNBS</td>
<td>9.95 ± 0.32</td>
<td>0.94 ± 0.03</td>
</tr>
<tr>
<td>Calcinet (0.2 μg/kg)</td>
<td>11.24 ± 0.20</td>
<td>0.96 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dex (0.6 mg/kg)</td>
<td>9.75 ± 0.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.98 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dex (0.6 mg/kg) + calcinert (0.2 μg/kg)</td>
<td>10.36 ± 0.26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.97 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> P < 0.01 vs. TNBS-treated mice.
<sup>b</sup> Nonsignificant vs. TNBS-treated mice.

### Table 2

Effect of dexamethasone and/or calcinert on clinical parameters of acute TNBS colitis

TNBS-treated mice were treated i.p. with dexamethasone and/or calcinert. BW change on day 3 in percentage of day 0, clinical activity score (CAS), colon length, and colon weight (distal 6 cm) were determined on day 5 as described in detail under Materials and Methods. Data represent mean ± S.E.M. from three separate experiments (eight mice per group per experiment).

<table>
<thead>
<tr>
<th></th>
<th>Δ BW</th>
<th>CAS</th>
<th>Colon Length</th>
<th>Colon Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.55 ± 0.34</td>
<td>0.45 ± 0.17</td>
<td>12.94 ± 0.16</td>
<td>150.29 ± 7.48</td>
</tr>
<tr>
<td>TNBS</td>
<td>-16.11 ± 1.60</td>
<td>11.50 ± 0.67</td>
<td>9.93 ± 0.15</td>
<td>327.41 ± 7.85</td>
</tr>
<tr>
<td>Calciner (0.2 μg/kg)</td>
<td>-14.42 ± 1.71&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.86 ± 0.86&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.00 ± 0.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>258.43 ± 11.96&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dex (0.6 mg/kg)</td>
<td>-10.60 ± 2.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.00 ± 1.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.91 ± 0.28&lt;sup&gt;b&lt;/sup&gt;</td>
<td>245.33 ± 7.17</td>
</tr>
<tr>
<td>Dex (0.6 mg/kg) + calcinert (0.2 μg/kg)</td>
<td>-7.38 ± 1.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.00 ± 0.60&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.32 ± 0.30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>210.75 ± 9.40&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dex (0.9 mg/kg)</td>
<td>-9.20 ± 1.99&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.11 ± 0.48&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.10 ± 0.39&lt;sup&gt;b&lt;/sup&gt;</td>
<td>228.44 ± 8.08&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dex (0.9 mg/kg) + calcinert (0.2 μg/kg)</td>
<td>-5.72 ± 0.66&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.45 ± 0.31&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.95 ± 0.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>171.55 ± 5.61&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dex (1.2 mg/kg)</td>
<td>-6.80 ± 0.82&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.88 ± 0.35&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.00 ± 0.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>179.75 ± 8.98&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dex (1.2 mg/kg) + calcinert (0.2 μg/kg)</td>
<td>-5.05 ± 1.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.90 ± 0.75&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.14 ± 0.24&lt;sup&gt;b&lt;/sup&gt;</td>
<td>166.56 ± 7.37&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> P < 0.05 vs. TNBS-treated mice.
<sup>b</sup> P < 0.01 vs. TNBS-treated mice.
<sup>c</sup> P < 0.001 vs. TNBS-treated mice.

### Statistical Analysis

All data are expressed as mean ± S.E.M. Clinical activity score of colitis and macroscopic and histological scores were statistically analyzed using the Mann-Whitney U test. Differences in parametric data were determined by the unpaired two-tailed Student's t test (SigmaStat, Chicago, IL). Differences were considered statistically significant with P < 0.05.

### Results

**Blood Calcium and Creatinine Profile.** We measured serum calcium and creatinine levels in mice following treatment with calcinert and/or dexamethasone. Although there was a trend toward hypercalcemia in mice treated with calcinert alone in comparison with controls, the treatment with dexamethasone alone and in combination with calcinert did not cause any significant alterations of serum calcium and creatinine levels (Table 1).

**Impact of Calcitriol and/or Dexamethasone on Inflammation and Colitis Severity in Acute and Established Ongoing Th1-Mediated TNBS Colitis.** The TNBS instillation in 45% ethanol led to a substantial wasting disease caused by severe diarrhea. Dexamethasone dose dependent (0.6–1.2 mg/kg) led to an improvement of colitis severity. Calcitriol on its own significantly reduced the severity of ongoing TNBS colitis. However, the most clinical benefit with respect to colitis severity was observed...
following the combined application of dexamethasone and calcitriol, as reflected by weight gain, as well as improvement of clinical, macroscopic, microscopic, and immunological parameters of colitis (Table 2).

Macroscopic inspections of colons resected 3 days after administration of the TNBS enema revealed a striking hyperemia, inflammation, and necrosis compared with the ethanol-treated control groups, which showed only negligible signs of inflammation (Fig. 1, A and B). Strikingly, the combined application of the steroids caused a synergistic improvement, with clinical disease scores falling to 20% of those of the TNBS group. This was associated with a substantial decrease of colitis-mediated hyperemia and inflammation.

Histological examinations (Fig. 1, C and D) determined by day 3, in the TNBS group, a transmural inflammation characterized by infiltration of inflammatory cells, predominantly lymphocytes and neutrophils that were associated with ulcerations, loss of goblet cells, and fibrosis throughout the entire colons. The combination of calcitriol and dexamethasone most effectively restored the normal histological appearance of the mucosa and submucosa compared with the TNBS group and the ethanol-treated control group (Fig. 1, C and D). Moreover, when applied 2 days after onset, the combination of dexamethasone and calcitriol still was very potent to ameliorate disease, as indicated by macroscopic and clinical assessment of colitis severity (Fig. 2, A–H; Table 3).

Fig. 1. Macroscopic and microscopic analysis of colons from mice with acute TNBS colitis. Mice were treated i.p. with calcitriol (0.2 µg/kg) and/or dexamethasone (0.6 mg/kg), respectively. A, representative photograph of colons from day 3 after the induction of TNBS colitis. 1, ethanol-treated control; 2, TNBS-treated; 3, TNBS + 0.2 µg/kg calcitriol; 4, TNBS + 0.6 mg/kg dexamethasone; 5, TNBS + 0.2 µg/kg calcitriol + 0.6 mg/kg dexamethasone. B, macroscopic score of colitis. Results are the mean ± S.E.M. from eight mice per group. C, photomicrographs of colon sections after treatment with 45% ethanol (C1), TNBS in 45% ethanol (C2), TNBS + 0.2 µg/kg calcitriol (C3), TNBS + 0.6 mg/kg dexamethasone (C4), or TNBS + 0.2 µg/kg calcitriol + 0.6 mg/kg dexamethasone (C5). D, histopathologic scoring. Original magnifications, 250×. Results are the mean ± S.E.M. from eight mice per group. *, P < 0.05; **, P < 0.01; ***, P < 0.001 versus TNBS-treated mice.
Calcitriol Affects the Differentiation of T Cells in Mice with TNBS Colitis. To integrate our clinical observations following calcitriol treatment with the possible molecular targets and mechanisms in a first set of experiments, we focused on the differentiation of T cells induced by vitamin D. We observed a clear-cut down-regulation of the inflammatory response with a combined steroid treatment including calcitriol (Table 4).

Calcitriol Inhibits the Th1 and Promotes the Th2 Profile in Mice with TNBS Colitis. Th1 lymphocyte differentiation is known to be based on a sequence of cell-intrinsic and exogenous, DC-derived factors, including aug-

TABLE 3
Effect of dexamethasone and/or calcitriol on the immune response of established TNBS colitis
TNBS-treated mice were treated i.p. with dexamethasone and/or calcitriol from days 3 to 5. MPO activity, TNF-α, and IL-12p70 were determined on day 5. The results are the mean ± S.E.M. from eight mice per group.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MPO Activity</th>
<th>TNF-α</th>
<th>IL-12p70</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.23 ± 0.18</td>
<td>3.17 ± 0.47</td>
<td>3.83 ± 0.20</td>
</tr>
<tr>
<td>TNBS</td>
<td>17.38 ± 1.20</td>
<td>16.77 ± 0.91</td>
<td>19.26 ± 0.75</td>
</tr>
<tr>
<td>Calcitriol (0.2 μg/kg)</td>
<td>10.58 ± 0.57 a</td>
<td>9.61 ± 0.96 a</td>
<td>13.72 ± 0.97 b</td>
</tr>
<tr>
<td>DEX (0.6 mg/kg)</td>
<td>8.84 ± 0.49 a</td>
<td>8.54 ± 0.64 a</td>
<td>10.50 ± 0.52 a</td>
</tr>
<tr>
<td>DEX (0.6 mg/kg) + calcitriol</td>
<td>3.65 ± 0.89 a</td>
<td>5.03 ± 0.73 a</td>
<td>7.04 ± 0.72 a</td>
</tr>
</tbody>
</table>

a P < 0.001 vs. TNBS-treated mice.
b P < 0.01 vs. TNBS-treated mice.
Calcitriol Enhances Regulatory T Cell Functions. Recent investigations clearly indicated that the suppressor cytokines IL-10 and TGFβ are involved in the control of colitis. Herein, calcitriol monotherapy significantly up-regulated IL-10 as well as TGFβ levels, whereas dexamethasone treatment alone failed to cause a significant induction of these cytokines (P < 0.01 versus dexamethasone, Fig. 3C and D). In contrast, and further supporting an independent role of calcitriol on GATA3 protein expression, dexamethasone alone at 0.6 mg/kg caused no significant reduction of T-bet protein expression compared with calcitriol and dexamethasone monotherapy (P < 0.001 versus dexamethasone, Fig. 3E). These results were supported by the finding that already calcitriol alone led to a significant 5-fold induction of GATA3 protein expression, whereas the combination of dexamethasone and calcitriol caused a significant 5-fold increase of GATA3 protein expression (P < 0.001 versus dexamethasone, Fig. 3F). In contrast, further supporting an independent role of calcitriol on GATA3 protein expression, dexamethasone alone at 0.6 mg/kg caused no significant GATA3 up-regulation.

Calcitriol Affects DC Mediators Responsible for a Proinflammatory Differentiation of T Cells in Mice with TNBS Colitis. To better understand the background of our results obtained so far in this colitis model, we expanded our investigations to the DC mediators IL-12p70 and IL-23p19, which play a pivotal role for the promotion of a proinflammatory differentiation of naive T cells toward Th1 and Th17, respectively. In mice with acute TNBS colitis, IL-12p70 expression was significantly up-regulated. Here, both calcitriol and dexamethasone, when applied as monotherapy, led to a distinct reduction of IL-12p70 production. However, again, the most prominent reduction of IL-12p70 was observed with a combination of dexamethasone and calcitriol (P < 0.01 versus dexamethasone; Fig. 5). For the first time in acute TNBS colitis, we observed an increased expression of IL-23p19. Protein expression of IL-23p19 was clearly inhibited following treatment with calcitriol and/or dexamethasone. In this setting, calcitriol was even more potent to down-regulate IL-23p19 expression, and we observed a significant difference between dexamethasone and calcitriol monotherapy with respect to their inhibitory potential on IL-23p19 (P < 0.05 dexamethasone versus calcitriol monotherapy). Moreover, the combined application of calcitriol and dexamethasone most effectively reduced IL-23p19 expression (Fig. 6A and B).

A recent investigation revealed that unlike IL-23, IL-6 and TGFβ in combination may be even more potent to induce the differentiation of pathogenic Th17 effector cells. In this study, IL-6 was significantly up-regulated in acute ongoing TNBS colitis. Both dexamethasone and calcitriol significantly down-regulated IL-6, whereas the calcitriol/dexamethasone combination resulted in an strong inhibition of IL-6 expression (Fig. 6E). Th17 effector cells are highly pathogenic and play a pivotal role in the maintenance of inflammation and in mediating tissue destruction. Here, Western blots of IL-17 revealed that, as hinted by calcitriol and dexamethasone combinatorial effects on IL-23p19 and IL-6 before,
this treatment exhibited the highest inhibitory activity on IL-17 expression in acute TNBS colitis (Fig. 6, C and D).

**Discussion**

Extending two previous investigations (Daniel et al., 2005, 2006), the data of this in vivo study in an accepted model of Th1-mediated colitis in mice clearly indicates and unravels that combining dexamethasone with calcitriol represents an attractive new immune modulatory treatment regimen of inflammatory disorders, including CD. To our knowledge, here we show for the first time that the complex immune-modulating effects of calcitriol may result from a differential down-regulation of proinflammatory capacities of intestinal DC as assessed by analysis of IL-12p70 as well as IL-23p19. This in turn leads to a moderate induction of a Th2 profile with increased levels of IL-4, which might already counteract Th1 and Th17 effector functions. Remarkably, however, calcitriol on its own, and more effective in combination with dexamethasone, substantially promoted Treg functions with distinct increases of the regulatory set of IL-10, TGFβ, FoxP3, and CTLA4.

Multiple epidemiological studies have indicated that a compromised vitamin D status is not only a well known risk factor for skeletal disorders but also contributes to the pathogenesis of frequent malignant, infectious, chronic inflammatory, and autoimmune disorders (e.g., insulin-dependent diabetes mellitus, inflammatory bowel disease, and multiple sclerosis). Especially among patients with CD, vitamin D...
deficiency is common, even when the disease is in remission (Cantorna et al., 2004). Furthermore, VDR deficiency (knockout) was shown to result in severe inflammation of the gastrointestinal tract, pointing toward the prominent role for VDR signaling in the regulation of gut inflammation (Froicu et al., 2003). In the absence of VDR, Th1 cell-driven IBD is more severe, and Th2 cell-driven asthma does not develop (Cantorna et al., 2004). The VDR gene maps to a region on chromosome 12 that has been shown to be linked to IBD by genome screening techniques (Simmons et al., 2000). Analysis of single nucleotide polymorphisms in VDR typed in patients with CD provides preliminary evidence for a genetic association between CD susceptibility and the VDR gene lying within one of the candidate regions determined by linkage analysis (Simmons et al., 2000).

The results provided by our study using a combination of calcitriol and dexamethasone demonstrate a potent inhibition of the DC-derived IL-12p70 followed by a prominent down-regulation of the Th1 inflammatory cytokines profiles in Th1-mediated TNBS colitis. To further support these observations, the combined application of calcitriol and dexamethasone used in our study most evidently led to a down-regulation of T-bet expression, whereas GATA3 protein expression was significantly up-regulated.

To expand the complexity beyond the Th1-Th2 paradigm, IL-23 comprising the p40 subunit of IL-12 and a specific p19 subunit was identified with the p40-p19 complex being secreted by activated DC and macrophages. Initially, only IL-23 was described to be responsible for IL-17 production and subsequent inflammatory diseases including the main-
Recent studies using mice with a reporter for FoxP3 mRNA also indicated that TGFβ may directly influence FoxP3 (Ziegler, 2006). Here, the combined application of calcitriol and dexamethasone enhanced FoxP3 expression accompanied by the above-discussed induction of IL-10 and TGFβ. However, in vitro-generated homogenous populations of IL-10 Tregs obtained by stimulating naive CD4+ T cells in the presence of a combination of the anti-inflammatory drugs vitamin D₃ and dexamethasone were shown not to express high levels of FoxP3 (Vieira et al., 2004). Thus, although FoxP3 appears to be important for the development and function of naturally occurring CD4⁺CD25⁺ T cells, in vitro-derived IL-10-secreting Tregs appear to have regulatory functions despite low levels of FoxP3. It seems also important to consider that in vivo Tregs might also be generated via interactions with tolerogenic DC in the periphery.

Here, we report a high expression of FoxP3 following combined application of dexamethasone and calcitriol, arguing that FoxP3 may be involved in multiple lineages of Tregs and can be induced depending on microenvironmental signals such as TGFβ. Supporting this concept, it was recently demonstrated that GC treatment also promotes or initiates the differentiation of effector Tregs in a FoxP3-dependent manner. In addition, this study demonstrates that FoxP3 and IL-10 are functionally linked together in vivo (Karagiannidis et al., 2004). These observations were further underlined by the fact that IL-10 in turn regulates responsiveness to GC. In CD4⁺ T cells, cultures isolated from GC-resistant asthma patients' addition of calcitriol and dexamethasone enhanced IL-10 synthesis to levels observed in GC-sensitive patients, thus restoring GC responsiveness (Xystrakis et al., 2006). Calcitriol significantly counteracted the reduction of GR expression following dexamethasone, and IL-10 up-regulated IL-10 expression; this increase was even more potent using the combination of dexamethasone and calcitriol, arguing a potential indirect benefit of these treatment regimens because these results suggest that the balance of TNFα/IL-10 during the course of an inflammatory disease may determine changes in sensitivity to GC. In CD patients, not only GR resistance often represents a critical healthcare problem, but also decreased calcitriol levels seem to trigger the inflammatory status (Cantorna et al., 2004; Peterlik and Cross, 2005). Thus, our results stress that the combined application of dexamethasone and calcitriol may be an appealing future therapeutic option carrying the additional benefit of a restored GC responsiveness, an induction of Tregs, and the production of IL-10.

Finally, calcitriol led to a considerable increase in CTLA4 expression; this increase was even more potent using the combination of calcitriol and dexamethasone. This supports the concept of an inductive potential of calcitriol on Treg functions. These findings are in line with recent reports indicating that CTLA4 engagement can up-regulate IL-10.

Fig. 5. Mice were treated i.p. from days 0 to 3 following the TNBS instillation with calcitriol (0.2 μg/kg) and/or dexamethasone (0.6 mg/kg), respectively. IL-12p70 production in colon protein extracts on day 3. The results are the mean ± S.E.M. from three different experiments (eight mice per group). Bars, mean ± S.E.M. of three separate experiments (n = 6) per group. *, P < 0.05; **, P < 0.01; ***, P < 0.001 versus TNBS-treated mice.

tenance and expansion of mucosal inflammation. However, subsequently, a mixture of IL-6 and TGFβ was found to be a potent cocktail, resulting also in the generation of IL-17-producing T cells (Harrington et al., 2005; Hunter, 2005; Bettelli et al., 2007). Remarkably, recent studies toward an orphan retinoid X receptor-related nuclear receptor, retinoid-related orphan receptor gamma, possibly interacting with VDR, revealed an essential IL-6-dependent function for the IL-17 expression of lamina propria T cells (Ivanov et al., 2006). Thus, IL-17-producing CD4⁺ effector cells differentiate along a distinct developmental program and might be antagonized by cytokines produced by Th1 or Th2 cells (in-
and TGFβ. Furthermore, FoxP3 has been shown to upregulate CTLA4 expression (Liu et al., 2003; Zheng et al., 2006). Thus, calcitriol may trigger a TGFβ/CTLA4/FoxP3-positive loop vital for the generation and maintenance of CD4⁺CD25⁺ Tregs in corticosteroid-treated mice with Th1-mediated colitis (Zheng et al., 2006).

In conclusion, we have shown in this mouse model of human CD that calcitriol functions as a promising new therapeutic option for Th1- or Th17-driven IBD, especially when combined with GC. Nevertheless, further studies are needed to assess whether the immune modulatory effects of calcitriol and dexamethasone combinations are indeed synergistic. The beneficial pharmacological profile of calcitriol might help to overcome the clinical refractoriness to GC therapy that is a common feature among IBD patients. Furthermore, its future therapeutic potential might not only result from its complex immunosuppressive capacities on proinflammatory signals of DC also leading to the observed inhibition of Th1...
and Th17 effector functions and the distinct promotion of the Th2 profile but might additionally lead to a long-term, highly appreciated induction of regulatory T cell functions, including IL-10 synthesis. These new data regarding the calcitriol activity carefully evaluated in an approved model of CD and supported by two previous investigations may accelerate preparations for a large prospective study with a calcitriol comedication, especially in CD patients with low active vitamin D₃ levels. Such a study may be facilitated by the advent of calcitriol analogs with an activity skewed more than 2 logs toward immune modulation, thus lowering the risk of a perturbation of calcium/phosphate and bone metabolism. With careful preparation, it should be feasible to design a study with meaningful primary and secondary endpoints to the benefit of the CD patients and to solve the ongoing enigma of calcitriol’s immune action.

References


Address correspondence to: Dr. Jürgen Stein, FEBG, First Department of Internal Medicine, Division of Gastroenterology and Clinical Nutrition, Johann Wolfgang Goethe University, Theodor-Stern-Kai 7, 60590 Frankfurt, Germany. E-mail: J.Steie@em.uni-frankfurt.de