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Predictive validity and immune cell involvement in the pathogenesis of piroxicam-accelerated colitis in interleukin-10 knockout mice

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ABSTRACT

Piroxicam administration is a method for induction of enterocolitis in interleukin-10 knockout (IL-10 k.o.) mice. The piroxicam-accelerated colitis (PAC) IL-10 k.o. model combines a dysregulated immune response against the gut microbiota with a decreased mucosal integrity. The predictive validity and pathogenic mechanisms of the model have not been thoroughly investigated. In this study, IL-10 k.o. mice received piroxicam in the chow, and model qualification was performed by examining the efficacy of prophylactic anti-IL-12/23p40 monoclonal antibody (mAb), anti-TNFα mAb, cyclosporine A (CsA) and oral prednisolone treatment. To evaluate cell involvement in the disease pathogenesis, specific cell subsets were depleted by treatment with anti-CD4 mAb, anti-CD8 mAb or clodronate-encapsulated liposomes. T cell receptor co-stimulation was blocked by CTLA4-Ig. Cytokine profiling ELISAs and calprotectin immunohistochemistry were performed on colon tissue. Treatments with anti-IL-12/23p40 mAb and CsA prevented disease in PAC IL-10 k.o. mice and reduced IFNγ, IL-17A, MPO and calprotectin levels in colon. Anti-TNFα mAb treatment caused amelioration of selected clinical parameters. No effect of prednisolone was detected. Depletion of $CD8^+$ cells tended to increase mortality, whereas treatment with anti-CD4 mAb or CTLA4-Ig had no significant effect on disease development. Clodronate liposome treatment induced a loss of body weight; nevertheless macrophage depletion was associated with a significant reduction in colonic pathology. In conclusion, reference drugs with known efficacy in severe inflammatory bowel disease were efficacious in the PAC IL-10 k.o. model. Our data indicate that in this model macrophages are a main driver of colitis, whereas $CD4^+$ cells are not.

1. Introduction

Inflammatory bowel disease (IBD) is a chronic, disabling disorder of the gastrointestinal tract, hypothesized to be caused by environmental and genetic factors and a subsequent dysregulated immune response against the gut microbiota [1–3]. Despite comprehensive research there is a distinct lack of knowledge about the disease and an unmet need for effective therapeutics exists. Based on anatomical and histological features IBD is subdivided into ulcerative colitis (UC) and Crohn's disease (CD). UC is characterised by continuous inflammation confined to the mucosa of rectum and colon. In contrast, the inflammation of CD is patchy and transmural. CD can affect the entire gastrointestinal tract [4,5], but 96% of CD patients have lesions in ileum and/or colon [6]. The therapeutic approach for induction and maintaining of remission is based on the individual patient profile. First line therapy includes 5-aminosalicylic acid and oral corticosteroids, but in patients with moderate or severe disease these drugs can be substituted by immunomodulators such as thiopurine analogues, methotrexate and antibodies neutralising tumour necrosis factor-alpha (TNF-α) [7–9]. Surgery is an option in severe IBD with stricturing and penetrating complications. In UC patients calcineurin inhibitors, like cyclosporine and tacrolimus, are alternative drugs for induction of remission in severe refractory cases, non-responsive to first line medication [7,9,10]. Still, current IBD treatment protocols have potential adverse side effects [8,11] and only 17% of UC [12] and 10% of CD patients [13] remain in remission ten years after diagnosis. Thus, there is a clear need for novel efficacious therapies in IBD.

Suitable animal models are an indispensable part of investigating disease pathogenesis and drug development. The capability to extrapolate knowledge from animal to human depends on the translational value of the model, including similarities in clinical features and pathogenic mechanisms, as well as its ability to identify drugs with therapeutic value in humans. Piroxicam accelerated colitis in interleukin-10 knockout (PAC IL-10 k.o.) mice is an in vivo model mimicking several features of human IBD, in particularly CD [14–16]. The PAC IL-10 k.o.

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model develops histopathological changes in both colon and ileum, characterised by mononuclear cell infiltration and hyperplasia of tunica mucosa. As in humans [17], disease development in PAC IL-10 k.o. mice requires a dysregulated immune response, a compromised mucosa barrier and commensal bacteria antigens in the gut lumen [15]. However, the underlying pathogenic mechanisms have not been thoroughly investigated. Monoclonal antibody (mAb) against the IL-12/23p40 subunit has proved clinical effect in CD patients [18,19], and we have previously demonstrated that anti-IL-12/23p40 mAb ameliorates colitis in PAC IL-10 k.o. mice [15]. In addition, broad-spectrum antibiotics [15,20], adenosine deaminase inhibition [21] and proteolytic bromelain [22] attenuate established colitis in the model. But the efficacy of drugs approved, or in late phase clinical development, for IBD has not yet been evaluated. In this study, enterocolitis of PAC IL-10 k.o. mice was treated prophylactically with anti-IL-12/23p40 mAb, anti-TNFα mAb, cyclosporine A (CsA) or oral prednisolone in order to access the predictive validity of the model. To elucidate the pathophysiological role of IBD-relevant immune cell subsets $CD4^+$ cells, $CD8^+$ cells and macrophages were depleted in the PAC IL-10 k.o. model and effector T cell receptor co-stimulation was blocked by a fusion protein, CTLA4-Ig. Furthermore, cytokine profiling ELISAs and calprotectin immunohistochemistry were performed on colon tissue.

2. Materials and methods

The experiments were conducted in accordance with the European Communities Council Directive 2010/63/EU for the protection of animals used for experimental purposes and approved by the Danish Animal Experiments Inspectorate, Ministry of Food, Agriculture and Fisheries, Denmark, as well as the internal Ethical Review Committee at Novo Nordisk A/S.

2.1. Animals

IL-10 k.o. mice backcrossed to a C57BL/6J background were purchased from Charles River Laboratories (Sulzfeld, Germany) in accordance with a licence agreement with MCG (Munich, Germany). 8–12 week old female mice were used in the experiments. The mice were housed under barrier protected conditions, free of agents listed in FELASA guidelines [23], with 10 mice per cage and a 12-hour light/dark cycle. The bedding was changed weekly, and in order to ensure a homogenous microbial environment, dirty cage bedding was transferred between cages. The mice had unrestricted access to water and piroxicam (Sigma-Aldrich, Broendby, Denmark) 200 ppm homogenized in 1324 Altromin diet (Altromin, Lage, Germany) from day 0 of the experiment. Weight change, faeces consistency and blood in faeces were examined three times weekly and used as measures of the clinical status of the mice. Hidden blood in the stool was detected by Hemoccult sensa cards® (Beckman Coulter, Birkeroed, Denmark). The stool and faecal blood scores were evaluated using Table 1, and the three clinical scores were accumulated in a composite disease activity index (DAI) score, modified from Murthy et al. [24]. Mice were sacrificed by cervical dislocation when severe disease was observed. Severe disease was defined as a weight loss exceeding 20% of the initial weight, max score in faeces consistency and faecal blood for more than two succeeding observations or a morbid appearance. Mice sacrificed within three days of planned take down were included in the post mortem analyses, whereas only mice alive throughout the whole study period were included in area under the curve (AUC) calculations.

Table 1
Scoring system for the three clinical parameters; weight loss, faeces consistency and faecal blood. Disease activity index (DAI) score is the accumulated score of the three individual parameters.

Weight loss	Score	Faeces consistency	Score	Faecal blood	Score
<1%	0	Hard and firm	0	Negative	0
1–5%	1	Firm but sticky	1	Hidden	1
5–10%	2	Soft but still coherent	2	Visual	2
10–15%	3	Soft with loss of shape	3	Rectal bleeding	3
15–20%	4	Liquid no firm consistency	4		

2.2. Prophylactic treatment of piroxicam-accelerated colitis in IL-10 k.o. mice

In the prophylactic treatment studies IL-10 k.o. mice received piroxicam chow throughout the study periods and the studies were terminated, when the respective control groups exceeded a mean weight loss of approximately 7%. PAC IL-10 k.o. mice were treated with neutralising rat anti-mouse TNFα mAb (25 or 5 mg/kg, clone XT3.11, n = 12) or isotype rat IgG1 (25 mg/kg, clone HRPN, n = 12) intraperitoneally (i.p.) three times a week, days 0–10. Rat anti-mouse IL-12/23p40 mAb (25 mg/kg, clone C17.8, n = 9) or isotype rat IgG2a (25 mg/kg, clone 2A3, n = 11) were administrated i.p. three times a week, days 0–15. All antibodies were purchased from Bio X Cell (West Lebanon, NH) and were tested for endotoxin content. A cohort of PAC IL-10 k.o. mice was treated with CsA (30 or 10 mg/kg, n = 11/10, Novartis, Copenhagen, Denmark) or sterile NaCl (n = 11) subcutaneously five times weekly, days 0–16. Prednisolone (3 or 1 mg/kg, n = 8; Sigma-Aldrich, Broendby, Denmark) or 0.5% carboxymethylcellulose sodium (n = 7; Sigma-Aldrich, Broendby, Denmark) were administrated by oral gavage once daily, days 0–14. The administrated doses were based on internal experience and published data [25–28].

2.3. Cell involvement in piroxicam-accelerated colitis in IL-10 k.o. mice

PAC IL-10 k.o. mice were treated with neutralising rat anti-mouse CD4 mAb (clone GK1.5, n = 11), rat anti-mouse CD8 mAb (clone YTS 169.4, n = 11) or isotype rat IgG2b (clone LTF-2, n = 9) i.p. three times a week, days −2 to 16. First depletion dose was 1 mg/mouse; subsequently doses were 0.5 mg/mouse. All antibodies were purchased from Bio X Cell (West Lebanon, NH) and were tested for endotoxin content. FACS analyses of the blood were performed to check the effectiveness of the depletions. To study the role of T cell receptor co-stimulation CTLA4-Ig (Orencia, 10 mg/kg, n = 12, Orifarm, Odense, Denmark) or human IgG1-Fc (10 mg/kg, clone Human Fc-G1, n = 12, Orifarm, Odense, Denmark) were administrated i.p. three times a week, days 0–10. To deplete macrophages before disease onset (prophylactic) clodronate-encapsulated liposomes (1 mg/mouse, n = 14; Encapsula NanoSciences, Brentwood, TN) or control PBS-encapsulated liposomes (1 mg/mouse, n = 14; Encapsula NanoSciences, Brentwood, TN) were administrated i.p. twice a week, days −2 to 13. To deplete macrophages after disease onset (interventive) clodronate-encapsulated liposomes (1 mg/mouse, n = 10; Encapsula NanoSciences, Brentwood, TN) or PBS-encapsulated liposomes (1 mg/mouse n = 10; Encapsula NanoSciences, Brentwood, TN) were administrated i.p. twice a week, from day 12. On day 12 the control group exceeded a mean weight loss of 7% and piroxicam treatment was discontinued. The interventive study was terminated at day 17. FACS analysis of single cell suspensions from the spleens was performed to check the depletion of macrophages. All administrated doses were based on internal experience and published data [29].

2.4. Hematology

Blood was obtained by *vena facialis* puncture. The blood was processed for serum or plasma samples as previously described [15], and stored at −80° until haptoglobin analyses were performed. Haptoglobin levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Life Diagnostics, West Chester, PA) according to the manufacturer's instructions with samples diluted 1:25000 times. The optical density in each well was read at 450 nm with a SpectraMax