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Depletion of regulatory T cells leads to an exacerbation of delayed-type hypersensitivity arthritis in C57BL/6 mice that can be counteracted by IL-17 blockade

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ABSTRACT

Rodent models of arthritis have been extensively used in the elucidation of rheumatoid arthritis (RA) pathogenesis and are instrumental in the development of therapeutic strategies. Here we utilise delayed-type hypersensitivity arthritis (DTHA), a model in C57BL/6 mice affecting one paw with synchronised onset, 100% penetrance and low variation. We investigate the role of regulatory T cells (Treg) in DTHA through selective depletion of Treg, and the role of IL-17 in connection with Treg depletion. Given the relevance of Treg in RA, and the possibility of developing Treg-directed therapies, this approach could be relevant for advancing the understanding of Treg in inflammatory arthritis. Selective depletion of Treg was achieved using a Foxp3-DTR-eGFP mouse, which expresses the diphtheria toxin receptor (DTR) and enhanced green fluorescent protein (eGFP) under control of the Foxp3 gene. Anti-IL-17 monoclonal antibody (mAb) was used for IL-17 blockade. Numbers and activation of Treg increased in the paw and its draining lymph node in DTHA, and depletion of Treg resulted in exacerbation of disease as shown by increased paw swelling, increased infiltration of inflammatory cells, increased bone remodelling and increased production of inflammatory mediators, as well as increased production of anti-citullinated protein antibodies. Anti-IL-17 mAb treatment demonstrated that IL-17 is important for disease severity in both the presence and absence of Treg, and that IL-17 blockade is able to rescue mice from the exacerbated disease caused by Treg depletion and caused a reduction in RANKL, IL-6 and the number of neutrophils. We show that Treg are important for the containment of inflammation and bone remodelling in DTHA. To our knowledge, this is the first study using the Foxp3-DTR-eGFP mouse on a C57BL/6 background for Treg depletion in an arthritis model, and we here demonstrate the usefulness of the approach to study the role of Treg and IL-17 in arthritis.

KEY WORDS: Rheumatoid arthritis, Regulatory T cells, IL-17, Neutrophils, C57BL/6, ACPA, Microbiota

INTRODUCTION

Rodent models of arthritis have been extensively used in the process of elucidating the pathogenesis of rheumatoid arthritis (RA), a chronic inflammatory disease characterised by chronic joint inflammation and bone erosion, and they are instrumental in the development of new therapeutic strategies (Asquith et al., 2009). Delayed-type hypersensitivity arthritis (DTHA) is an arthritis model in C57BL/6 (B6) mice that is characterised by synchronised onset, 100% penetrance and low variation. DTHA is induced by modifying a protein-antigen-induced delayed-type hypersensitivity response in the paw by administration of an anti-type-II-collagen antibody cocktail (anti-CII) between immunisation and challenge. The result is severe inflammatory arthritis affecting one, predefined, hind paw (Atkinson et al., 2012). Notably, the anti-CII dose required for DTHA is five- to eight-times lower than required for induction of collagen-antibody-induced arthritis (CAIA) in B6 mice (Hutamekalin et al., 2009).

Owing to their well-established role in controlling inflammation in many animal models, we hypothesise that regulatory T cells (Treg) play a role in the resolution of inflammation in DTHA. It has been shown in mouse models of arthritis that deficiency or depletion of Treg exacerbates disease, and increasing numbers of Treg can reduce disease activity (Gizinski and Fox, 2014; Kelchtermans et al., 2009; Miyara et al., 2014; Noack and Miossec, 2014; Zaiss et al., 2010). The development of a more sustained disease phenotype in the absence of Treg would let us study disease drivers unchecked by this immunoregulatory cell subset and to identify which disease driver mechanisms are suppressed by Treg in vivo in experimental arthritis. The purpose of the present study was therefore to investigate the mechanisms of self-limiting disease in DTHA through selective depletion of Treg after disease induction. Treg depletion studies in experimental arthritis models are scant and have mainly used the anti-CD25 approach. Anti-CD25 treatment in collagen-induced arthritis (CIA) accelerates disease (Kelchtermans et al., 2005; Morgan et al., 2003). Administration of anti-CD25 prior to induction exacerbates glucose-6-phosphate isomerase (G6PI)-induced arthritis (Frey et al., 2010) and antigen-induced arthritis (AIA) (Frey et al., 2005). However, using anti-CD25 antibodies for depletion of Treg also targets effector T cells (Teff). The Foxp3-DTR-eGFP mouse allows selective depletion of
The Tregs without affecting Teff. This mouse expresses a fusion of a diphtheria toxin receptor (DTR) and enhanced green fluorescent protein (eGFP) under the control of the forkhead box protein 3 (Foxp3) gene locus, which means that Tregs can be selectively depleted by administration of diphtheria toxin (DT) (Feuerer et al., 2009; Lalji et al., 2007). Treg depletion in Foxp3-DTR-eGFP mice on a DBA/1 background exacerbated G6PL-induced arthritis (Irmler et al., 2014). However, to our knowledge, this is the first study to address, in-depth, the use of the Foxp3-DTR-eGFP mouse for Treg depletion in experimental arthritis and the first to use the Foxp3-DTR-eGFP mouse on a B6 background in experimental arthritis.

RA has recently been associated with changes in the gut microbiota (Scher et al., 2013; Zhang et al., 2015). In spontaneous mouse models of autoimmune arthritis, joint inflammation is attenuated under germ-free conditions, but colonisation of the gut with commensal microbes is sufficient to elicit joint inflammation comparable to that observed in conventional mice (Abdollahi-Roodsaz et al., 2008; Wu et al., 2010). In these models, the colonisation of the gut resulted in a perturbed Treg/Teff balance, which was associated with disease onset and progression. The interplay between Tregs and the gut microbiota modulates Treg abundance and function and might thereby also affect onset and progression of arthritis. Therefore, we also analysed the faecal microbiota following DTHA induction alone and in conjunction with Treg depletion.

In the present study, we found that depleting Tregs after onset of DTHA led to an exacerbation of arthritis. Inflammatory cell infiltration, osteoclast activation and bone erosion were increased in Treg-depleted mice. Treg depletion also increased levels of anti-mutated-citrullinated-vimentin (MCV) antibodies, indicating an increase in autoimmunity associated with increased protein citrullination. Production of a wide range of cytokines and chemokines was increased, including IL-17, a cytokine involved in the pathogenesis of both RA and murine experimental arthritis. We show that treating Treg-depleted mice with anti-IL-17 monoclonal antibody (mAb) rescues them from exacerbation of disease through a reduction of circulating neutrophils and a reduction in IL-6 and receptor-activator of nuclear factor κB ligand (RANKL).

RESULTS

A highly activated and proliferating subset of Tregs is found in the lymph node draining the arthritic paw early after disease onset

We investigated the dynamics of Tregs in DTHA by analysing the popliteal lymph node draining the arthritic paw (dPLN), the popliteal lymph node draining the control paw (ndPLN), paw infiltrate and blood at different time points during disease by flow cytometry (Fig. 1A). Tregs were defined as live CD45+TCRβ+CD4+CD25+FoxP3+ cells and Teff cells by inverting the CD25+FoxP3+ gate within the CD45+TCRβ+CD4+ gate (full gating strategy shown in Fig. S1A). Tregs were most abundant in the dPLN on day 2 and 4 after DTHA induction and were still elevated compared to day 1 and naive mice on day 7. The increase in Treg number in the dPLN was also reflected in blood (Fig. 1B). A dramatic increase in activated (CD44+) and proliferating (Ki67+) Tregs was seen in the dPLN, but not in the ndPLN or in blood (Fig. 1B, bottom graphs). Analysis of Foxp3 mRNA showed a significantly higher Foxp3 expression in paws on day 3 and 8 compared to before arthritis induction (Fig. 1C). The same was seen with Cilia4 mRNA, which codes for the surface marker cytotoxic T-lymphocyte-associated protein 4 (CTLA4) found on Tregs and memory T cells, but to a greater extent on Treg numbers in blood or popliteal lymph nodes was observed during the immunisation phase of DTHA (from naive to day 0), so we also analysed the inguinal lymph nodes (ILNs), which drain the immunisation sites, in naive mice and immediately prior to methylated bovine serum albumin (mBSA) challenge. In these lymph nodes a dramatic increase in the frequency of both Treg and Teff cells was observed (Fig. 1D), but this increase was not reflected in the number of circulating Tregs or Teff on day 0 (Fig. 1B). Interestingly, a subpopulation of CD25+FoxP3hi Tregs was found in the dPLN but not in the ndPLN or blood (Fig. 1E,F). These CD25+FoxP3hi Tregs were found to possess an activated phenotype and were actively proliferating already from day 1 post-DTHA induction (Fig. 1G). We also analysed cells isolated from the arthritic paw on day 2 and 4 after DTHA induction and found the fraction of Tregs of total T cells to increase from 12.17±1.09% to 14.70±0.63% from day 2 to 4 (mean±s.e.m., P=0.079, Student’s t-test). Analysis of immune cells isolated from non-inflamed paws is not feasible owing to the very low number of cells that is possible to harvest. We also found subpopulations of CD25hiFoxP3hi and Ki67+ Tregs in the paw. Among paw infiltrating T cells on day 2 and day 4, we found 33.72±4.16% and 23.04±1.55% of Tregs to be CD25hiFoxP3hi (mean±s.e.m., P=0.044, Student’s t-test) and 82.16±1.3% and 86.35±1.2% of Tregs to be Ki67+ (mean±s.e.m., P=0.034, Student’s t-test), respectively. In summary, we observed increased numbers of activated and proliferating Tregs in the dPLN and paw early after mBSA challenge in DTHA.

Depletion of Tregs after arthritis induction exacerbates inflammation and leads to a rapid increase in arthritic disease activity

In order to assess the importance of Tregs in regulating DTHA severity, we selectively depleted Tregs by administration of DT at 24 and 48 h after arthritis induction to Foxp3-DTR-eGFP mice (Foxp3-DTR+ littermates (Foxp3-DTR−) and littermate controls (Foxp3-DTR−)). This resulted in depletion of all eGFP+ cells in Foxp3-DTR+ mice as confirmed by flow cytometry (Fig. S2). However, eGFP+ cells eventually repopulate the Treg niche after transient DT treatment, as shown in Fig. S2C. Depletion of Tregs rapidly led to increased paw and ankle swelling, which persisted up to at least day 10 (Fig. 2A,B). In Treg-depleted mice, levels of the acute-phase protein serum amyloid P component (SAP) were elevated in serum on day 10 compared to control mice (Fig. 2C), as was C-terminal telopeptide of type I collagen (CTX-I; Fig. 2D), whereas tartrate-resistant acid phosphatase (TRAP) serum levels were not increased (Fig. 2D). Histopathological analysis on day 10 showed increased infiltration of macrophages into the Treg-depleted paws (Fig. 2E,F). However, inflammation and new bone formation at extra-articular sites were increased in Treg-depleted mice, and so was the histopathological sum score (Fig. 2H). Interestingly, we observed a correlation (r=0.5949, P=0.0092, n=18) between extra-articular inflammation and new bone formation (Fig. 2I), indicating that new bone formation in DTHA is an inflammation-associated process.

Depletion of Tregs leads to an increase in biomarkers of arthritis and inflammatory cells in the affected paw and in circulation

Next, we investigated the mechanisms of the increased arthritis response following Treg depletion by sampling on day 4 and 7 post-arthritis induction. We found increased paw swelling in Treg-
depleted mice (Fig. 3A), and increased levels of SAP, IL-6 and matrix metalloproteinase 3 (MMP3) on both day 4 and 7 in Treg-depleted mice (Fig. 3B). Levels of CTX-I were increased on day 7 but not day 4. Neutrophil chemoattractant granulocyte colony stimulating factor (G-CSF) was increased on day 4, but not day 7 (Fig. 3B). Interestingly, we detected production of anti-MCV in DTHA, and, following Treg depletion, anti-MCV antibody levels were increased in serum (Fig. 3B). In order to investigate whether Treg play a role in regulating bone resorption in DTHA, we measured levels of RANKL, osteoprotegerin (OPG) and TRAP in whole paw tissue. We found that levels of both RANKL and TRAP increased in the arthritic paws on day 4 and 7, and the RANKL/OPG ratio increased after Treg depletion (Fig. 3C). We also investigated the cellular players in the increased inflammatory response after Treg depletion. Systemic neutrophilia and increased numbers of neutrophils in inflamed paws and dPLNs were observed.
Increased numbers of macrophages in the inflamed paws and dPLNs were also observed (Fig. 4B and data not shown). Numbers of CD4+ T cells in the paw were increased compared to controls on day 7 but not day 4 (Fig. 4B), but there was no increase in CD4+ T cells in the dPLN (Fig. 4C). However, CD4+ T cells in the dPLN were found to be more activated (Fig. 4D). Taken together, these data show that depletion of Tregs increases systemic inflammation, increases the production of autoantibodies, increases bone resorption, increases the numbers of neutrophils and macrophages in the inflamed paw and its draining lymph node, and enhances T-cell activation.

Concomitant depletion of Tregs and blockade of IL-10 signalling increased paw and ankle swelling to an even larger degree than Treg depletion or IL-10 blockade alone. We found that concomitant depletion of Tregs and blockade of IL-10 signalling increased paw and ankle swelling to an even larger degree than Treg depletion or IL-10 blockade alone (Fig. S3A-C). Active
bone remodelling on day 10 post-arthritis induction, measured through serum CTX-I, was only detectable in mice with both depleted Tregs and blocked IL-10R signalling (Fig. S3D).

**Depletion of T<sub>regs</sub> increases bacterial diversity transiently without affecting the overall major taxonomic composition of fecal microbiota**

Fecal samples from the distal colon were collected before DTHA induction on day 0, and on day 4 and 9 following DTHA induction with or without concurrent depletion of T<sub>regs</sub>. The composition of the microbiota was investigated by sequencing of the V4 hypervariable region of the 16S rRNA gene. Principal coordinate analysis (PCoA) at the level of operational taxonomic units (OTUs) revealed no significant changes in overall clustering following DTHA induction (Fig. S4). However, we found that the bacterial composition of the T<sub>reg</sub>-depleted mice was more diverse on day 4 compared to the non-depleted mice (Fig. 5A), with a tendency towards an increase in alpha diversity for the T<sub>reg</sub>-depleted mice from day 0 to 4 (P=0.141), whereas beta diversity did not differ significantly (Fig. 5B). Interestingly, alpha diversity correlated inversely with ankle swelling for the T<sub>reg</sub>-depleted group at day 4 (Spearman’s ρ=−0.964; P<0.01; n=7). Analysis at the phylum level revealed no significant differences between the two groups, or between days within each group (Fig. 5C). We detected no significant changes in the relative abundance of genera (Fig. 5D) between groups, or between days within each group, although the abundance of *Lactobacillus* tended to be higher on day 4 compared to day 0 and 9 (P=0.084 and 0.12 for FoxP3-DTR<sup>+</sup> and P=0.229 and 0.305 for FoxP3-DTR<sup>−</sup>, respectively). Correlations between taxa abundance and ankle swelling, paw swelling, TRAP, and CTX-I, respectively, did not show high correlation coefficients or patterns of correlations (data not shown). Taken together, these data show that DTHA induction alone or in conjunction with the depletion of T<sub>reg</sub> induce only minor and transient compositional changes in the fecal bacterial microbiota. Thus, contrasting with results obtained by analysis of RA patients (Zhang et al., 2015), no significant arthritis-associated changes in the gut microbiota were detected in the mouse models.
Depletion of Tregs leads to increased production of inflammatory mediators locally in the arthritic paws

To further elucidate the mechanisms of increased inflammation following Treg depletion in DTHA, we measured levels of a range of cytokines and chemokines in whole paw tissue. The cytokines IL-1β, IL-17, IL-12(p70), IL-6 and IL-10 were all increased in paw tissue on both day 4 and 7 compared with controls (Fig. 6). IFNγ was increased only on day 4 and TNFα only on day 7 (Fig. 6). The chemokines CCL2 (MCP-1), CCL10 (IP-10), CCL5 (LIX), CCL5 (RANTES) and CXCL9 (MIG) were all increased in paw tissue on both day 4 and 7 after DTHA induction by flow cytometry. CD4+ T cells were defined as live CD45+CD11b+Ly6G+. Mean±s.e.m. shown, n=10. Student’s t-test, *P<0.05; **P<0.01. (B) Cell counts of total live cells, CD4+ T cells, macrophages and neutrophils in the arthritic paw analysed on day 4 and 7 after DTHA induction by flow cytometry. CD4+ T cells and neutrophils defined as before, macrophages defined as live CD45+CD11b+FcγRII+ cells. Mean±s.e.m. shown, n=10. Student’s t-test, *P<0.05; **P<0.01; ***P<0.001. (C) Counts and percentages of CD4+ T cells analysed in the dPLN on day 4 and 7 after DTHA induction by flow cytometry. CD4+ T cells were defined as live CD45+TCRβ+CD4+ cells. Mean±s.e.m. shown, n=10. Student’s t-test, **P<0.01. (D) Median fluorescence intensity (MFI) of CD44-APC (left) and CD62L-PE (right) in the CD4+ T-cell gate. Mean±s.e.m. shown, n=10. Student’s t-test, **P<0.01; ***P<0.001. Results presented in this figure are representative of two independent experiments. Full flow cytometry gating strategy is shown in Fig. S1B,C.

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Treatment with anti-IL-17 mAb rescues mice from exacerbation of disease caused by depletion of Tregs

Based on our findings so far, we hypothesised that IL-17 could play a part in the increased disease activity observed following Treg depletion. To test this, we set up a study where Treg-depleted mice were also treated with anti-IL-17 mAb. We found that anti-IL-17 treatment reduced paw swelling in mice both with and without intact Treg compartments (Fig. 7A). We also found that anti-IL-17 treatment of Treg-depleted mice reduced paw swelling to levels seen in isotype-treated, non-Treg-depleted mice, thus rescuing them from the exacerbated disease caused by the depletion of Tregs.
(Fig. 7A). Starting anti-IL-17 treatment at immunisation (Fig. S5) did not reduce paw swelling to a greater degree than anti-IL-17 treatment begun at the time of arthritis induction (Fig. 7A). Anti-IL-17 treatment did not have an effect on the production of anti-MCV (Fig. 7B). There was a tendency towards reduced myeloperoxidase (MPO) levels in anti-IL-17-treated mice both with and without intact Treg compartments, compared with isotype-control-treated animals (Fig. 7C). In addition, neutrophil numbers in circulation were reduced after IL-17 blockade in mice both with and without an intact Treg compartment (Fig. 7D). We found that blockade of IL-17 reduced levels of IL-6, CXCL1, CXCL5 and G-CSF in arthritic paws of mice with intact Treg compartments and tended to reduce IL-6 and CXCL1 in mice depleted of Tregs (Fig. 7E). IL-17 blockade reduced the levels of IL-6 and CXCL1 in mice that received Treg depletion + anti-IL-17 to levels seen in non-Treg-depleted + isotype-treated mice (Fig. 7E). Importantly, anti-IL-17 treatment reduced levels of RANKL in paw tissue in mice both with and without intact Treg compartments (Fig. 7F). In line with this, there was a tendency towards a reduction of TRAP levels after IL-17 blockade. Treg depletion reduced the RANKL/OPG ratio and IL-17 blockade increased the RANKL/OPG ratio, indicating that Treg depletion favours a more resorptive state, whereas IL-17 blockade reverses the effect of Treg depletion on bone erosion (Fig. 7F). In summary, we find that IL-17 blockade rescues mice from the increased disease activity observed after Treg depletion, and does so through a reduction in neutrophil numbers and local IL-6, CXCL1 and RANKL levels.

**DISCUSSION**

In RA, dysregulation of the Treg compartment has been reported, and Treg numbers and levels of IL-17 expression correlate with disease severity (Noack and Miossec, 2014; Zaiss et al., 2010). Different approaches to tipping the Teff/Treg balance towards Treg dominance have been put forward as potential therapeutic strategies in RA and other inflammatory diseases; however, none have yet been fully successful (Miyara et al., 2014). Knowledge of the roles played by Tregs in experimental arthritis models is instrumental for the development of safe and effective therapeutic strategies involving Tregs in humans. In this study, we investigated the kinetics of Tregs in DTHA and characterised the exacerbated inflammatory response that resulted from selective depletion of Tregs. To our knowledge, this is the first study to characterise the mechanisms of exacerbated disease in an arthritis model following depletion of Tregs using Foxp3-DTR-eGFP mice. Transient DT treatment, although sufficient to give the desired biological effect in many cases, can also leave a residual number of eGFP+ FoxP3+ cells, and these cells eventually repopulate the Treg niche (Lahl et al., 2007; Lahl and Sparwasser, 2011). However, because we were interested in examining the effects of Treg depletion on inflammation in the early stages of DTHA, our assessment was that this mouse was suitable for our purpose, despite its limitations.

Treg numbers in DTHA expanded rapidly and these cells were found to be highly activated and proliferating in lymphoid and paw tissue. This indicates that Tregs are important in the early stages of DTHA. However, the fact that we observed severe inflammation in the affected paw despite the expansion of Tregs could be the result of a dysregulation of the Th17/Treg balance, because increased Th17 activity is also seen in the early stages of DTHA (Atkinson et al., 2012). In support of this, we also show in the present study that neutralisation of IL-17 reduced severity of disease and could prevent the detrimental effects of Treg depletion in the model.

Selective Treg depletion increased arthritis severity, and the observed correlation between paw inflammation and new bone formation indicated that new bone formation could be linked to inflammation in DTHA. In support of this, we have previously
shown that suppression of inflammation in DTHA with anti-TNFα antibodies (Atkinson et al., 2012) or anti-C5aR antibodies (Atkinson et al., 2015) could reduce bone formation. Inflammation has also been linked to bone formation in the CIA, proteoglycan-induced arthritis (PGIA) and SKG inflammatory arthritis models (Denninger et al., 2015; Schett et al., 2009; Haynes et al., 2012; Keller et al., 2012), and in ankylosing spondylitis (Maksymowych et al., 2013; Wanders et al., 2005). However, Treg depletion also led to increased bone resorption in DTHA in the present study. The presence of these two opposing phenotypes can seem counterintuitive, but the bone formation and bone erosion take place at different areas of the paw (Atkinson et al., 2012), and simultaneous bone erosion and bone formation has also been observed in the CIA model in a recent study (Denninger et al., 2015). The net effect, however, is a negative association between inflammation and bone mass, as previously described (Gilbert et al., 2013; Proulx et al., 2007; Walsh et al., 2005).

Depletion of Tregs led to increased CD4+ T-cell activation and to increased recruitment of CD4+ T cells to the site of inflammation. This shows that the control of Teff activation in the dPLN and Teff numbers in the paw is one of the mechanisms of Treg-mediated suppression of DTHA. We also observed increased levels of anti-MCV, demonstrating an augmented immune response to a citrullinated self-antigen. Anti-MCV antibodies and other anti-citrullinated protein antibodies (ACPAs) are used as prognostic biomarkers of disease and joint erosion in RA, and ACPA levels correlate with serum CTX-I in RA patients (Gudmann et al., 2015; Jilani and Mackworth-Young, 2015). The fact that we observe a break of self-tolerance to MCV in DTHA and that anti-MCV levels increase with increased disease severity adds to the strengths of DTHA as a model of RA. However, the current study did not address which, if any, role anti-MCV plays in the pathogenesis of DTHA.

Previous studies have shown that Tregs are able to limit the accumulation of Th17 cells at inflammatory sites and draining lymph nodes (Lohr et al., 2006). Increased IL-17 production was observed in the Treg-depleted mice in the present study. This is supported by the observed increase in production of IL-6, IL-23 and IL-1β, which are cytokines that favour Th17 polarisation (Lubberts et al., 2005;
Fig. 7. Treatment with anti-IL-17 rescues mice from exacerbated disease caused by Treg depletion. (A) Anti-IL-17 treatment was initiated at the time of arthritis induction 1 day prior to Treg depletion and given every 48 h until experiment termination on day 7. Left graph: paw swelling day 0-7. Right graph: area under curve (AUC) of paw swelling day 0-7. ns, not significant, *P ≤ 0.05, ***P ≤ 0.001. (B) Levels of anti-mutated-citrullinated-vimentin (MCV) antibodies in serum measured by ELISA as optical density (OD). Dotted line represents levels in n=5 naïve mice. (C) Levels of myeloperoxidase (MPO) in paw tissue measured by ELISA of whole paw homogenate supernatants. (D) Neutrophils/ml blood measured by flow cytometry. Neutrophils were defined as live CD45+TCRβ−CD11b+Ly6G+ cells. ns, not significant, *P ≤ 0.05. (E) Analysis of inflammatory mediators in whole paw homogenate supernatants from Treg-depleted mice (blue bars) and non-depleted mice (black bars) treated with anti-IL-17 mAb or isotype control, using multiplex analysis [IL-1β, TNFα, IFNγ, IL-6, IL-12(p70), MIP-2 (CXCL2), IP-10 (CXCL10), RANTES (CCL5), MIG (CXCL9), MIP-1α (CCL3), KC (CXCL1), G-CSF and GM-CSF] and ELISA (IL-23). Mean±s.e.m. shown, n=10. ns, not significant, *P ≤ 0.05. (F) Levels of RANKL, OPG and TRAP were measured in whole paw homogenate supernatants from Treg-depleted mice (blue) and non-depleted mice (black) treated with anti-IL-17 mAb or isotype control, using ELISA. Dotted line represents mean OPG level in n=2 naïve mice. CCL, CC chemokine ligand; MIP, macrophage inflammatory protein; CXCL, CXC chemokine ligand; IP, interferon gamma-induced protein; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; MIG, monokine induced by gamma interferon; OPG, osteoprotegerin; RANKL, receptor-activator of nuclear factor κB ligand; RANTES, regulated on activation, normal T cell expressed and secreted; TRAP, tartrate-resistant acid phosphatase.
Sondereregger et al., 2008; Sutton et al., 2006). IL-17 is an important cytokine in experimental arthritis when T cells play a part in the pathogenesis (Atkinson et al., 2012; Lubberts et al., 2005). Blockade of IL-17 has previously been shown to ameliorate experimental arthritis and is also a promising therapeutic avenue in RA (Lubberts et al., 2001, 2004; Föllinger et al., 2011; van den Berg and McInnes, 2013). We found that treatment with anti-IL-17 could attenuate disease in DTHA, and the results indicate that IL-17 is more important for DTHA pathogenesis after the antigen challenge. The increased IL-17 production after Treg depletion could also contribute to the increased bone erosion observed, and results from other studies support this. IL-17 induces osteoclastogenesis from human monocytes in the absence of RANKL (Yago et al., 2009) and upregulates RANKL expression (Gizinski and Fox, 2014), and blocking IL-17 prevents joint destruction in CIA through an increased RANKL/OPG ratio (Lubberts et al., 2004). Our results thus suggest that the increase in bone erosion could be driven by an increase in the RANKL/OPG ratio driven by increased IL-17 activity.

We observed increased IL-10 production in arthritic paws after Treg depletion, which suggests that the contribution of IL-10 by Tregs to immunoregulation in DTHA is negligible. In a study in which Tregs were depleted using anti-CD25 mAb, we found that concomitant blockade of IL-10 signalling increased paw and ankle swelling to an even larger degree than Treg depletion or IL-10 blockade alone. In the absence of Tregs, regulatory B cells and their release of IL-10 could represent a compensatory regulatory pathway. B-cell-derived IL-10 has been shown to regulate inflammation in other mouse models (Fillatreau et al., 2002; Mauri et al., 2003; Mizoguchi et al., 1997). In experimental autoimmune encephalitis (EAE), components from Mycobacterium tuberculosis in the complete Freund’s adjuvant (CFA) that is used to induce disease provide Toll-like receptor (TLR) agonists that can trigger regulatory functions of B cells that are required for the resolution of EAE (Lampropoulou et al., 2008).

Because B cells are not required for the induction of DTHA (Tanaka et al., 2007) and because CFA containing M. tuberculosis is also used for induction, it could be speculated that B cells indeed play an immunoregulatory role in DTHA and that depletion of B cells on top of Treg depletion would further exacerbate DTHA.

We also observed an increased number of neutrophils in paws, dPLNs and blood as well as increased production of granulopoietic factors and neutrophil chemoattractants after Treg depletion. This could in part be driven by the increased IL-17 production, because IL-17 is known to stimulate neutrophil recruitment through induction of G-CSF, GM-CSF and chemokatrantcs, including CXCL1 (Hamilton, 2008; Kolls and Lindén, 2004; Park et al., 2005; Schwarzenberger et al., 2000). Increased levels of CXCL1 and CXCL2 at the site of inflammation have also previously been shown to result from Treg depletion in mice (Richards et al., 2010). Indeed, blockade of IL-17 reduced neutrophil numbers in Treg-depleted mice and reduced levels of CXCL1. IL-6 production can be induced by IL-17 (Kolls and Lindén, 2004) and, in line with this, we observed a major decrease of IL-6 production following IL-17 blockade. IL-6 plays an important role in neutrophil trafficking (Fielding et al., 2008; Lally et al., 2005), so the observed decrease in blood neutrophil numbers after IL-17 blockade could be a result of the decreased IL-6 levels. Taken together, these data support a regulatory role for IL-17 in neutrophil recruitment through IL-6 and CXCL1.

Recent findings have suggested an association between the gut microbiota and RA (Scher et al., 2013; Zhang et al., 2015), and interactions between the gut microbiota and immune function, including Treg, neutrophils and IL-17, are well established (Maynard et al., 2012). However, in our model, depletion of Tregs did not significantly change the bacterial composition, indicating that immune-driven changes in the gut microbiota are unlikely to play an important role in disease progression in our model. We observed a tendency towards higher alpha diversity in Treg-depleted mice and that alpha diversity correlated inversely with ankle size at day 4. Interestingly, we observed a tendency towards an increased abundance of Lactobacillus on day 4, which also correlated with maximal ankle swelling. Lactobacillus has been shown to be over-represented in RA patients compared to healthy subjects (Liu et al., 2013; Zhang et al., 2015), and the abundance of Lactobacillus was also shown to correlate with disease activity in RA patients. Furthermore, mono-colonisation with Lactobacillus bifidus was shown to be sufficient for inducing joint inflammation in a mouse model of spontaneous arthritis via TLR2- and TLR4-dependent signalling affecting the balance between Treg, Th17 and Th1 in the gut (Abdollahi-Roodsaz et al., 2008). When comparing the microbial changes observed in laboratory animals to humans, it is important to consider the marked environmental differences. Moreover, in DTHA, severe disease is observed after just a few days, whereas RA develops over several years. Although the composition of the microbiota can change within days, the short duration of the model might not be sufficient to markedly affect the composition of the gut microbiota in a manner that reflects the situation in human disease pathogenesis. Nevertheless, the findings suggest a link between Lactobacillus and joint inflammation both in rodent models and RA patients.

**Conclusion**

Taken together, our findings show that Tregs are important for the containment of inflammation in DTHA, and that Treg depletion in DTHA acts, at least partly, through increasing IL-17 and RANKL activity and through increasing numbers of neutrophils in blood, dPLNs and paws. We show that IL-17 drives an increase in inflammation in the absence of Tregs: administration of anti-IL-17 mAb just prior to Treg depletion rescues mice from the exacerbation of disease associated with Treg depletion through a reduction in IL-6 and CXCL1 production, reduced neutrophil numbers and a reduction in the RANKL/OPG ratio. Thus, an imbalance in the Treg and Th17 relationship could be a driver of inflammation in DTHA. To our knowledge, this is the first study using the Foxp3-DTR-eGFP mouse on a C57BL/6 (B6) background for Treg depletion in an arthritis model, and we here demonstrate the usefulness of the approach and build upon the already existing body of work in this new model. Many genetically modified mice are bred and maintained at Forschungseinrichtungen für Genetik (Bremen, Germany) and can thus be combined with any other experimental approach. As a result, the Foxp3-DTR-eGFP model facilitates a better understanding of the role of Tregs in an arthritis model, and we here demonstrate that the approach can be used to further increase the understanding of disease pathways. However, the limitations of the Foxp3-DTR-eGFP mouse generated by BAC transgenesis must be kept in mind when choosing this model system. Given the relevance of Tregs in RA and the possibility of developing Treg-mediated therapies for RA, using this approach for selective depletion of Tregs could yield important results to help understand the diverse roles of Tregs in RA and other inflammatory diseases.

**MATERIALS AND METHODS**

**Mice**

Female C57BL/6J mice were purchased from Taconic, Ry, Denmark. Female Foxp3-DTR-eGFP mice were generated as described in Lahl et al. (2007), and bred and maintained at Forschungseinrichtungen für Genetik (Bremen, Germany).
Experimentelle Medizin, Charité Universitätsmedizin Berlin or purchased from Jackson Laboratories (Bar Harbor, ME) on a NOD background (generated as described in Feuerer et al., 2009) and backcrossed for at least eight generations onto the C57BL/6 background at Taconic, Ry, Denmark. Foxp3-DTR-eGFP mice express a fusion protein consisting of the diphtheria toxin (DT) receptor (DTR) and enhanced green fluorescent protein (eGFP) under the control of the Foxp3 gene locus inserted by BAC transgenesis. This construct allows selective depletion of Tregs by DT injection. Animals were housed in a facility with a 12-h light/dark cycle and with free access to water and standard rodent chow (Altromin®). All animal experiments performed in Germany were in accordance with the guideline 2010/63/EU from the European Union and the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes. Animal protocols were approved by the ethics committee and the Berlin state authorities (LAGeSo registration # G0331/08). All animal experiments performed in Denmark were conducted according to Danish legislation and have been approved by the Danish Animal Inspectorate and the Novo Nordisk ethical review board.

Induction and assessment of DTHA
Mice were anaesthetised by isoflurane/O₂:N₂O and immunised intradermally (i.d.) with methylated bovine serum albumin (mBSA) (Sigma, St Louis, MO) emulsified in complete Freund’s adjuvant (CFA) (Difco, Detroit, MI) at the tail base. Four days later, mice were given 1000 µg (approx. 50 mg/kg body weight) anti-mouse type II collagen antibody (anti-CII) cocktail (Chondrex, Redmond, WA) containing the clones A2-10 (IgG2a), F10-21 (IgG2a), D8-6 (IgG2a), D1-2G (IgG2b) and D2-112 (IgG2b) intravenously in 200 µl phosphate-buffered saline (PBS). Seven days after immunisation the mice were challenged with 200 µg mBSA subcutaneously in 20 µl PBS in the right foot pad. The left foot pad was given 20 µl PBS only and served as control. Baseline paw and ankle thickness measurements were made on the right paw on day 0 prior to mBSA challenge. Paw and ankle swelling was measured using a dial thickness gauge (Mitutoyo, Japan), and was calculated as right paw or ankle thickness minus baseline measurement.

Depletion of Tregs
Mice were injected intraperitoneally with 1 µg DT (Mercer, Darmstadt, Germany) in 100 µl PBS 24 and 48 h after DTHA induction. Depletion of Tregs was confirmed by flow cytometry on whole blood sampled 24 h after the final DT dose (Fig. S1 shows a representative depletion check). Cells were gated on TCRβ, CD4 and CD25 and analysed for expression of eGFP. Antibodies used were anti-CD16/32 (BD Biosciences, NJ) for blocking of Fc-receptor binding, anti-TCRβ-PerCP-Cy5.5, clone H57-597 (eBioscience, San Diego, CA), anti-CD25-APC, clone PC61.5 (eBioscience, San Diego, CA), anti-CD4-PE-Cy7, clone RM4-5 (BD Biosciences, NJ) or anti-CD4-Quill605 (Invitrogen, Carlsbad, CA).

Depletion of Tregs and IL-10R blockade
For depletion of Tregs, mice were dosed with 200 µg rat anti-mouse CD25 mAb (clone PC61.5, BioXcell, West Lebanon, NH) or rat IgG1 anti-trinitrophenol (TNP) isotype control (Novo Nordisk A/S, Måløv, Denmark) 11 and 8 days prior to immunisation. For blockade of IL-10R, mice were dosed with 250 µg rat anti-mouse IL-10R mAb (clone 1B1.3A, BioXcell, West Lebanon, NH) or rat IgG1 anti-TNP isotype control (Novo Nordisk A/S, Måløv, Denmark) three times weekly from the time of immunisation. Depletion of Tregs was confirmed by flow cytometry on whole blood samples drawn 48 h after the second dose of anti-CD25. Mouse regulatory T cell staining kit (eBioscience, San Diego, CA) was used. Antibodies used were CD45-PerCP, CD4-APC and CD25-FITC all from BD Biosciences (NJ), and FoxP3-PE (eBioscience, San Diego, CA).

Anti-IL-17 mAB treatment
Mice were treated with 400 µg rat anti-mouse IL-17 mAB (clone 17F3, BioXcell, West Lebanon, NH) or rat IgG1 isotype control (clone MOPC-21, BioXcell, West Lebanon, NH) in 200 µl PBS on the day of arthritis induction and with 200 µg rat anti-mouse IL-17 mAB or isotype control in PBS after 48 h and every 48 h until the experiment was terminated on day 7. For results presented in Fig. S4, mice were dosed with 200 µg rat anti-mouse IL-17 mAB (clone 17F3, BioXcell, West Lebanon, NH) or rat IgG1 isotype control (clone MOPC-21, BioXcell, West Lebanon, NH) in 200 µl PBS from the time of immunisation and every 48 h until study termination.

Histopathology
Paws were processed and stained with hematoxylin and eosin (H&E), Safranin O and for tartrate-resistant acid phosphatase (TRAP) as previously described (Atkinson et al., 2012). TRAP stains osteoclasts red, and Safranin O stains cartilage red. The intensity of Safranin O staining is directly proportional to the proteoglycan content in cartilage. Pathological changes in the paws were assessed on H&E-, TRAP- and Safranin-O-stained sections. The extra-articular infiltration of inflammatory cells (assessed on a scale of 0–3) and arthritic changes were assessed separately. Arthritic changes were assessed on metatarsal and tarsal joints as synovitis, cartilage destruction and bone erosion, and scored separately on a 0-3 scale. For each of the three parameters of arthritic changes, an average between the two joint areas was calculated. In addition, new bone formation and extra-articular infiltration overall in the paw was scored on a 0-3 scale. New bone formation was defined as the presence of osteophytes, as described in Atkinson et al. (2012). The histology score was calculated by adding the five scores (extra-articular infiltration, synovitis, cartilage destruction, bone erosion and bone formation), whereas the extra-articular infiltration score is left out in the arthritis score. The person who performed the evaluation was blinded to the experimental setup.

Immunohistochemistry and digitalised image analysis
Immunohistochemical (IHC) detection of macrophages and neutrophils in the paws was performed as previously described (Atkinson et al., 2015). The sections were all digitally scanned and studied using a NanoZoomer Digital Pathology Virtual Slide Viewer (Hamamatsu Photonics, Shizuoka, Japan). Automated digital image analyses of the infiltrating macrophages (F4/80+) and neutrophils (Ly6B.2+) in the paws were performed using the Visiopharm Integrator System (VIS; version 4.2.2.0, Visiopharm, Hoersholm, Denmark). On individual digital images of the arthritic paw a region-of-interest (ROI) was automatically defined of the entire paw. The bone marrow was outlined manually and excluded from analysis, as was hair follicles and artefacts. Next, an analysis was run inside the ROI to detect the brown DAB (3-3′-diaminobenzidine-tetrahydrochloride) staining of the target cells. The results are given as percentage of tissue area positive for F4/80 or Ly6B.2 of the entire paw area.

Flow cytometry
Single-cell suspensions of blood, popliteal lymph nodes and paw infiltrate were prepared as previously described (Atkinson et al., 2012). All samples were subjected to Fc-blocking prior to antibody staining using anti-CD16/32 (BD Biosciences, NJ). Dead cells were excluded using Fixable Near-IR Vital dye (Invitrogen, Carlsbad, CA). Antibodies used were anti-CD45-V500 (clone 30-3F11, BD Biosciences, NJ), anti-CD45-eFluor450 (clone 30-3F11, eBioscience, San Diego, CA), anti-TCRβ-PerCP-Cy5.5 (clone H57-597, eBioscience, San Diego, CA), anti-CD11b-APC (clone M1/70, eBioscience, San Diego, CA), anti-CD8α-Pacific Blue (clone 53-6.7, Biolegend, San Diego, CA), anti-TCRβ-FITC (clone I3D1, eBioscience, San Diego, CA), anti-CD62L-PE (clone MEL-14, eBioscience, San Diego, CA), anti-CD44-APC (clone IM7, eBioscience, San Diego, CA), anti-CD8a-Pacific Blue (clone 53-6.7, Biolegend, San Diego, CA), anti-F4/80-eFluor450 (clone BM8, eBioscience, San Diego, CA), anti-Ly6-G-PE (clone 1A8, BD Biosciences, NJ) and anti-CD11b-APC (clone M1/70, eBioscience, San Diego, CA). Samples were run on an LSRII or FACSCanto flow cytometer and data were analysed using FACSDiva software (BD Biosciences, NJ). All cells were gated on live/dead marker and CD45 before further analysis. Neutrophils were defined as CD11b+Ly6G−, macrophages as CD11b+F4/80−, T cells as TCRβ+, and CD4 and CD8 T cells as TCRβ+CD4− and TCRβ+CD8−, respectively. Full gating strategies for the individual cell populations are shown in Fig. S1.
Multiplex analysis of inflammatory markers in paw homogenate

Hind paws were sampled at selected times after DTHA induction and each paw was placed in 1.25 ml of an ice-cold custom-made homogenization buffer containing a solution of 200 mM NaCl, 5 mM EDTA, 10 mM Tris, 10% glycerin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mg/ml leupeptin and 28 mg/ml aprotinin with a pH value of 7.4 (Ampliqon, Skovlunde, Denmark). The paws were homogenized by using a T25 Ultraturrax homogeniser (IKA, Staufen, Germany) followed by centrifugation at 10,000 g for 15 min. The supernatants were centrifuged and concentrated once more at 10,000 g for 15 min. The final supernatants were analysed undiluted for levels of IL-1β, TNFα, IFNγ, IL-6, IL-10, IL-17, IL-12(p40), MIP-2 (CXCL2), IP-10 (CXCL10), LIX (CXCL5), RANTES (CCL5), MIG (CXCL9), MIP-1α (CCL3), KC (CXCL1), MCP-1 (CCL2), G-CSF and GM-CSF using bead-based Luminox® xMAP® technology with Milliplex kits from Millipore (Billerica, MA) according to the manufacturer’s instructions. For statistical analysis, any values below the detection limit were set to the detection limit for the analyte in question and any values above the detection limit were set to the upper detection limit for the analyte in question.

Enzyme-linked immunosorbent assays (ELISAs)

Levels of serum amyloid P component (SAP) were measured in serum using kits from Genway (San Diego, CA) according to the manufacturer’s instructions. Levels of receptor-activator of nuclear factor kappa B (RANKL), osteoprotegerin (OPG) and IL-23 were measured in paw homogenate supernatants using kits from R&D Systems (Minneapolis, MN) according to the manufacturer’s instructions. Levels of CTX-I and TRAP were measured in serum and paw homogenate supernatants using ELISA kits from Immunodiagnostics Systems (Boldon, UK) according to the manufacturer’s instructions. Levels of MMP3, IL-6 and G-CSF were measured in serum using kits from R&D Systems (Minneapolis, MN) according to the manufacturer’s instructions. Antibodies to mutated citrullinated vimentin (anti-MCV) were measured using kits from Orgentec Diagnostika GmbH (Mainz, Germany) with the modification that the detection antibody supplied in the kit was substituted with goat anti-mouse IgG-HRP (Invitrogen, Carlsbad, CA) diluted 1:3000 and the substrate solution supplied was substituted with substrate reagents from R&D Systems (Minneapolis, MN).

mRNA deep sequencing

Arthritic hind paws were removed at the hairline and homogenised in Qiang RLT buffer (Qiagen, Germantown, MD) with 1% 2-betamercaptoethanol (Sigma-Aldrich, St Louis, MO) and stored at −80°C. The Ambion Magmax prep protocol was used to extract total RNA. RNA quality was assessed using the NanoDrop and RNA quality was assessed using the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNAseq libraries were prepared using the Illumina TruSeq Sample Prep Kit (Illumina, Inc., San Diego, CA). 120 ng of total RNA was used for input. Barcode adapters were added to samples in such a way as to allow pooling of samples in flow cell lanes in a randomized pattern relative to sample annotation and sorting. Samples were sequenced using an Illumina HiSeq 2000 (Illumina, Inc., San Diego, CA) at a multiplexing level sufficient to generate 10-million to 25-million reads per sample. Following generation of sequence, reads were aligned to the mouse genome version NCBI m37 using TopHat (http://tophat.cbcb.umd.edu/). Quality control of sequencing data was accomplished by using the ShortRead package in R (http://www.r-project.org/). Aligned reads were mapped to Ensembl transcript models and converted to reads per kilobase of transcript per million mapped reads (RPKM) values using Cufflinks (http:// cufflinks.cbcb.umd.edu/).

Bacterial 16s rDNA sequencing

Bacterial DNA from fecal samples from the distal colon was extracted using the Nucleospin soil kit (Macherey-Nagel, Düren, Germany) following the manufacturer’s protocol. Yield and integrity of the DNA were assessed by Nanodrop and agarose gel electrophoresis, respectively. 16s rDNA amplification and library generation were performed as previously described (Holm et al., 2015). PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA) and normalized to 16-21 ng/μl per sample. Subsequently, samples were pooled (5 μl of each sample) and quantified using a Qubit dsDNA HS Assay Kit and a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA). 14 pm library and 0.7 pm PhiX Control v3 (Illumina, San Diego, CA) were sequenced using an Illumina MiSeq V2 PE500 cartridge (500 cycles) on an Illumina MiSeq. Sequences were analysed using QIME v1.7.0 with default settings including quality-based sequence trimming, removal of primers, assembly of paired-end sequences and chimera checking (Caporaso et al., 2010). De novo operational taxonomic unit (OTU)-picking was performed by using UCLUST (Edgar, 2010) utilizing 97% sequence similarity. OTUs were assigned against the Greengenes database v11.2 (DeSantis et al., 2006) using the RDP-classifier (Wang et al., 2007) with an 80% confidence threshold. Analyses were performed in R v3.2.0 using the metagenomeSeq (Paulson et al., 2013), PhyloSeq (McMurdie and Holmes, 2013) and Vegan (Oksanen et al., 2011) packages. Data was filtered for low-abundant OTUs by removing OTUs present in fewer than three of the 42 samples and with a relative abundance across all samples ≤0.5%. Analyses on filtered data were performed with an average of 20,731±7655 (s.d.) sequences per sample compared to an initial 24,974±9382 (s.d.) sequences before filtering. Alpha diversity (Shannon index) was calculated on unfiltered data. Read counts were normalized using metagenomeSeq (Paulson et al., 2013), which utilizes a cumulative-sum scaling where raw counts are divided by the cumulative sum of counts up to a particular quantile. Principal coordinate analysis (PCoA) using Bray-Curtis dissimilarity indices, analysis of differential abundance of taxa and beta diversity analysis using Whittaker’s species turnover were performed on filtered and normalized data.

Statistics

Statistical analyses were conducted using GraphPad Prism software version 6.04. Non-parametric data or non-normal parametric data were analysed using the Mann–Whitney U-test, and parametric data were analysed using a two-sided unpaired Student’s t-test or one-way ANOVA. For statistical analysis of the histology score data, a two-sided unpaired Student’s t-test with Welch correction was used. For bacterial 16s rDNA, statistical analyses were conducted using R differences in abundance of phyla, alpha diversity measures and beta diversity between and within the FoxP3-DTR− and FoxP3-DTR+ groups over the course of the study using the Wilcoxon Rank-Sum and Wilcoxon Signed-Rank test, respectively, with Benjamini–Hochberg P-value adjustment. Adonis test of significance was performed using the calculated Bray–Curtis distance matrix, to test for overall differences between the FoxP3-DTR− and FoxP3-DTR+ groups and within each group over the course of the study. Correlation analyses between taxonomic abundances, alpha diversity, serum markers, and paw homogenate supernatants were performed in R using Spearman’s rank correlation test with Benjamini–Hochberg P-value adjustment. Differences between groups and correlation coefficients were considered significant when P≤0.05 and levels of significance were assigned as *P≤0.05, **P≤0.01 and ***P≤0.001. In Figs 6 and 7E P-values were corrected for mass significance using the False Discovery Rate method. The raw and corrected P-values are shown in Table S1.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

S.M.A. designed, performed and analysed the animal studies, immunoassays, flow cytometry, histopathology and immunohistochemistry, and drafted the manuscript. A.N. conceived the study, participated in its design and coordination, and provided intellectual support. U.H. and A.H. assisted in designing animal studies and provided intellectual support. E.B. and N.B.D.-S. performed the analysis of fecal microbiota.
composition, analysed the results and helped draft the manuscript. K.S., B.F. and K.Kruse performed and analysed the mRNA deep sequencing. K.Kristiansen, S.S. and C.H. helped draft the manuscript and provided intellectual support. All authors read and approved the final manuscript.

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