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RESEARCH LETTER

Prevention of diabetes by a hydrolysed casein-based diet in diabetes-prone BioBreeding rats does not involve restoration of the defective natural regulatory T cell function

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Abbreviations

BB	BioBreeding
DB	Diabetes prone
DR	Diabetes resistant
FOXP3	Forkhead box P3
HC	Hydrolysed casein

To the Editor: Diabetes-prone (DP)-BioBreeding (BB) rats show reduced natural regulatory T cell (nTreg; $CD4^+$/$CD25^+$/forkhead box P3 $[FOXP3]^+$) levels and function, and spontaneously develop type 1 diabetes from 70 days of age [1, 2]. Adoptive transfers of nTregs from diabetes-resistant (DR)-BB rats to DP-BB rats prevents diabetes in DP-BB rats [3, 4]. Environmental factors such as diet are critical triggers for the development of type 1 diabetes [5]. Using the DP-BB rat model for type 1 diabetes, we and others have shown that a hydrolysed casein (HC)-based diet reduces the incidence of diabetes from 90% to 50%, and delays the mean time of diabetes onset [5–8]. Reduced dietary diabetogenic triggers, skewing of immune responses and induction of islet neogenesis are thought to be the main mechanisms behind the effects of the HC-based diet [5–8].

In this study we investigated whether upregulation of nTreg capacity can also contribute to the prevention of type 1 diabetes by an HC-based diet in DP-BB rats.

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DR-BB and DP-BB rats were fed on the HC-based diet from weaning and were monitored for the development of diabetes. The conventional plant-based diet used was a standard laboratory rodent diet (Rmh-B2181; Hope-farms, Woerden, The Netherlands). The HC-based diet (TD99482; Harlan-Teklad Custom Research Diets, Madison, WI, USA) was a modification of the AIN-93G diet (Harlan-Teklad Custom Research Diets) containing 200 g/kg HC (as source of amino acids), 3 g/kg L-cysteine, 509.8 g/kg cornstarch, 120 g/kg sucrose, 70 g/kg soyabean oil, 50 g/kg cellulose, 35 g/kg mineral mix, 10 g/kg vitamin mix, 2 g/kg choline bitartrate and 0.20 g/kg butylated hydroxyanisole antioxidant. DP-BB rats were monitored until 130 days of age. Animals were weighed three times per week. In the case of weight loss, blood glucose was measured in tail vein blood using a glucose sensor (Reflolux S; Boehringer Mannheim, Mannheim, Germany). When blood glucose exceeded 15 mmol/l (non-fasting value), rats were considered diabetic and killed. The animals were derived from the Worcester DP-BB and DR-BB strain, but were maintained and bred at our institutional Central Animal Facility under specified pathogen-free and viral antibody-free conditions. The animals received humane care in compliance with the principles of laboratory animal care (National Institutes of Health publication no. 85-23; revised 1985) and the Dutch law on experimental animal care. The University Ethical Board for Animal Studies approved all animal experiments reported in this study.

Spleen and lymph nodes were obtained from rats on the standard or HC-based diet at 60 days of age (prediabetic), at diabetes onset (diabetic) and from non-diabetic rats at 130 days of age (protected). Lymph nodes and spleens were teased apart and passed through a 100 µm mesh nylon gauze. The cells were washed twice with culture medium (RPMI-1640; PAA Laboratories, Pasching, Austria) containing 25 mmol/l HEPES, 2 mmol/l L-glutamine, gentamycin and 10% (vol./vol.) FCS. Cell suspensions were stained with antibodies for αβ T cell receptor (clone R73; eBioscience, San Diego, CA, USA), CD4 (clone OX35; BD Bioscience, San Diego, CA, USA), CD25 (clone OX39; BD Bioscience) and FOXP3 (clone FJK-16 s; eBioscience). Cells were measured by flow cytometry on a FACS-Calibur (BD-Biosciences) and analysed using FlowJo software (TreeStar, Ashland, OR, USA).

$CD4^+CD25^-$ T cells and $CD4^+CD25^{high}$ T cells were sorted immediately after cell staining from the following combination: αβ T cell receptor/CD4/CD25. The populations were sorted using a high-speed cell sorter (MoFlo; Dako Cytomation, Fort Collins, CO, USA). FACS analysis showed that the $CD4^+CD25^{high}$ T cells were >90% FOXP3-positive.

To analyse $CD4^+CD25^{high}$ T cell-mediated suppression, 5×10^4 $CD4^+CD25^-$ (responder) T cells were co-cultured in round-bottom 96-well plates with 5×10^4 irradiated antigen-presenting cells, Concanavalin A (2.5 µg/ml), and purified $CD4^+CD25^{high}$ (nTregs cells) in a 1:1 ratio. In all experiments, responder cells were isolated from DR-BB rats. All cultures were conducted for 72 h at 37°C in the presence of 5% CO_2 and 95% air.

DP-BB rats showed reduced (absolute and relative) levels of nTregs in their mesenteric lymph nodes compared with DR-BB rats (Fig. 1a), confirming previous observations by our group and others [2–4]. Feeding the HC-based diet to DP-BB and DR-BB rats did not change nTreg levels (absolute and relative) in their mesenteric lymph nodes (Fig. 1a). In addition, the HC-based diet did not change levels of FOXP3 in the $CD4^+CD25^+$ T cell fraction. The same results were observed in the spleen (data not shown). Real-time RT-PCR did not show changes in *Foxp3* expression in ileum tissue of DP-BB rats receiving the HC-based diet (data not shown). This indicates that nTreg levels in the lamina propria are also not affected by the HC-based diet.

As shown in Fig. 1b, nTregs of DR-BB rats are anergic to polyclonal stimulation and suppress the proliferation of $CD4^+/CD25^-$ effector cells ($p<0.05$, Mann–Whitney *U* test). The nTregs of DP-BB rats on the standard diet proliferated better in response to polyclonal stimulation than those of DR-BB rats ($p<0.05$, Mann–Whitney *U* test) and were not able to suppress the proliferation of DR-BB effector cells. In a 1:1 ratio, we even observed enhanced proliferation ($p<0.05$, Mann–Whitney *U* test). The nTregs of DP-BB rats fed the HC-based diet did not differ in their proliferation and suppressive capacity compared with DP-BB rats fed on the standard diet (Fig. 1b).

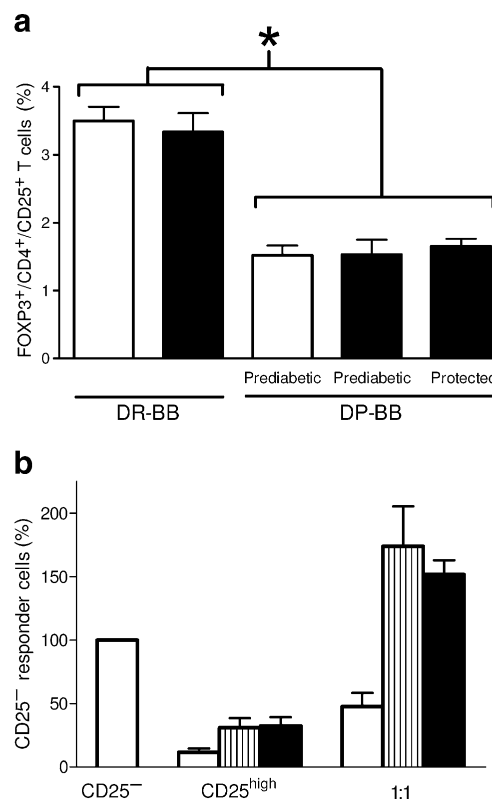

Fig. 1 Characterisation of naturally occurring regulatory T cells in DP-BB and DR-BB rats. **a** Levels of $CD4^+CD25^+FOXP3^+$ T cells (nTregs) were measured by flow cytometry and expressed as percentage (mean±SEM) of total lymph node cells. DR-BB and DP-BB rats were fed the standard rodent diet (white bars) or the HC-based diet (black bars) from 21 days of age. nTreg levels were analysed at 60 days of age (DR-BB and prediabetic DP-BB) or at 130 days of age (protected DP-BB). The absolute yield of nTregs from mesenteric lymph nodes in the different groups was as follows: DR-BB on standard diet $2.7\times10^6\pm0.15\times10^6$; DR-BB on HC diet $2.6\times10^6\pm0.21\times10^6$; prediabetic DP-BB on standard diet $3.0\times10^5\pm0.28\times10^5$; prediabetic DP-BB on HC-based diet $3.2\times10^5\pm0.47\times10^5$; and protected DP-BB on the HC-based diet $3.3\times10^5\pm0.22\times10^5$. Animals analysed per group, $n=5$. Groups were compared using the Mann–Whitney *U* test; *$p<0.05$. **b** Functionality of natural occurring regulatory T cells in BB rats. nTregs from DR-BB and DP-BB rats on a standard or a HC-based diet were isolated by cell sorting at 60 days of age (five animals per group) and subjected to a suppression assay in vitro. Cell culture was conducted as detailed in main text. The following $CD4^+CD25^{high}$:$CD4^+CD25^-$ ratios were tested: $CD4^+CD25^{high}$ T cells alone, 1:1 and $CD4^+CD25^-$ T cells alone. The results are expressed as per cent (mean±SEM) of $CD4^+CD25^-$ (responder) T cells. The average absolute counts per min of the responder cells was 782±184. Animals analysed per group: $n=5$. Groups were compared using the Mann–Whitney *U* test. White bars, DR-BB rats on standard rodent diet; striped bars, DP-BB rats on standard rodent diet; black bars, DP-BB rats on HC-based diet

In summary, these results further strengthen previous observations by our group and others [2–4] that the naturally occurring Tregs (characterised by $CD4^+/CD25^+/FOXP3^+$) in DP-BB rats are not functional. Moreover, these results show that the prevention of diabetes in the DP-BB rat model of type 1 diabetes by the HC-based diet is not caused by induction of the frequency and functionality of naturally occurring Tregs. These results suggest that the HC-based diet probably prevents diabetes development in the DP-BB rat by: (1) preventing direct activation of the auto-reactive T cell pool [5–7]; (2) skewing these cells to a less pathogenic phenotype [5–7]; and/or (3) the induction of islet neogenesis [8]. However, induction of other regulatory T cells in the $CD4^+CD25^-$ T cell fraction cannot be excluded. Future studies should therefore focus on modulating diabetes in the DP-BB rat model of type 1 diabetes by adoptive transfer of $CD4^+CD25^-$ T cells from BB rats treated with a HC-based diet.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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