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BIFIDOBACTERIA BbC50 FERMENTATION PRODUCTS INDUCE HUMAN CD4+ REGULATORY T CELLS WITH ANTIGEN-SPECIFIC ACTIVATION AND BYSTANDER SUPPRESSION

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Probiotic bacteria have been shown to have health benefits in various situations (inflammation, allergy, infection). We previously showed that a bacteria-free fermentation product of *Bifidobacterium breve* C50 (BbC50sn) induced high IL-10 secretion by human dendritic cells. As IL-10 is a regulatory cytokine, the aim of the present study was to examine whether DCs cultured in the presence of BbC50sn could induce regulatory T cells in an allogeneic context. Purified $CD4^+CD25^-$ human T cells were co-cultured with allogeneic BbC50sn-treated dendritic cells for 4 weeks. The T cell population (BbC50sn-T) was analysed both at phenotypical and functional [ability to inhibit a mixed lymphocyte reaction (MLR)] levels. We showed that T lymphocytes acquired phenotype characteristics of regulatory T cells after 4 weeks of co-culture with BbC50sn-DCs, and inhibited *in vitro* T lymphocyte proliferation and IFN-γ production in an MLR. Transwell experiments demonstrated that this suppressive activity was not T cell contact-dependent but probably mediated by a soluble factor. Although BbC50sn-T cells secreted significant amounts of IL-10 and TGF-β, their suppressive effect is most likely not mediated through these cytokines. This is, to our knowledge, the first demonstration of *in vitro* regulatory T cell induction by a bacteria-free fermentation product in an allogeneic context.

Dendritic cells (DCs) are professional antigen-presenting cells that have a key role in the immune response. These cells are present throughout the body, including the mucosa where they orchestrate innate and adaptive immune responses by means of their pattern recognition receptors such as Toll Like Receptors (TLR). DCs can either prime naïve T cells to elicit an effector immune response against infectious agents or promote immune tolerance to innocuous antigens such as food and commensal bacteria (1). A breakdown in commensal bacteria tolerance resulting from DC dysfunction in the

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gut has been shown to lead to the development of inflammatory bowel diseases (2). Also immune tolerance disruption of food and self-antigens leads to allergy (3) and autoimmune diseases (4), respectively.

The main effectors of tolerance are the regulatory T cells. These cells have an important role in controlling ongoing immune responses and silencing self-reactive T cells. Besides their critical role in the control of autoimmunity, they have been shown to regulate transplantation tolerance and allergy (5, 6). Regulatory T cells also have an important role in the gut where they maintain intestinal homeostasis (7). It was initially considered that when DCs were in an immature or a "tolerogenic" semi-mature state, they could drive the differentiation of regulatory T cells (8, 9). However, several studies have shown that regulatory T cells can also be induced by DCs displaying a mature phenotype (10).

Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" as referred by WHO/FAO expert's consultation. Several studies have shown a protective or curative effect of probiotic bacteria in colitis (11), inflammatory bowel diseases (12), allergy (13) and infection (14). Moreover, it has now become clear that probiotic bacteria directly influence host immunity. Many studies have shown that probiotics may confer an immune-modulatory function on DCs through induction of an anti-inflammatory or a tolerogenic profile (15, 16). A recent study described induction of regulatory T cells by certain probiotic strains (17). Interestingly, many of the health benefits seen with live bacteria were also obtained with dead cells (18). Indeed, it has been shown that dead bacteria can trigger a beneficial immune response in different animal models as well as in *in vitro* assays (18). Furthermore, certain studies have also demonstrated that probiotic bacteria can act on the immune system through the soluble factors produced during a fermentation process (18, 19). Until today, the use of probiotics was only envisaged in the treatment of allergy (21) and graft-versus-host (GVH) disease (22) but not in the case of organ grafts. The allogeneic response represents a strong Th1 response (23). Nevertheless, a body of evidence demonstrated that it could be inhibited by several regulatory ways such as regulatory T or B cells (24).

Soluble factors from *Bifidobacterium breve* C50 (BbC50) have been shown to have anti-inflammatory activities *in vitro* (25). Furthermore, we have previously shown that BbC50 supernatant (BbC50sn) could affect the maturation of DCs via the TLR-2 pathway (26). These DCs may contribute to a tolerogenic orientation of T cells as they secrete large amounts of IL-10. In this study, we further investigated the possibility of these BbC50sn-treated dendritic cells (BbC50sn-DCs) in generating *ex vivo* regulatory T cells in an allogeneic context.

MATERIALS AND METHODS

Reagents

RPMI1640, Foetal calf serum (FCS), L-Glutamin, Penicillin/Streptomycin and Phosphate-Buffered Saline (PBS) were obtained from Invitrogen (Gibco, France). FCS was decomplemented at 56°C for 30 min before use. Ficoll (Lymphoprep) was purchased from Axis-Shield (distributed by AbCys SA, France), recombinant human (rh) IL-2 and IL-4 from R&D systems (Lille, France) and rhGM-CSF from AbCys SA (Paris, France). LPS came from Sigma-Aldrich (Saint-Quentin Fallavier, France). BbC50sn was produced by Blédina SA (Steenvoorde, France) as previously described (26).

Antibodies

The following mouse anti-human monoclonal antibodies were used: anti-CD4-FITC, anti-CD69-FITC, anti-CD127-PE, anti-CD152 (CTLA-4)-PE and their respective isotype controls (Beckman Coulter/Immunotech), anti-CD25-APC (allophycocyanin), anti-CD39-APC, anti-CD134 (Ox40)-PE and their respective isotype controls (BD Biosciences/Pharmingen), and anti-GITR-PE and its isotype control (R&D systems). The Fix&Perm Cell permeabilisation kit (Caltag laboratories) was used for intracellular staining with the anti-CD152-PE antibody according to the manufacturer's instructions. Foxp3 staining was performed using the Foxp3 staining set (eBioscience) with the rat IgG anti-Foxp3-PE antibody (clone PCH101) and its isotype control according to the manufacturer's instructions.

Human monocyte-derived dendritic cells

Blood from healthy volunteer donors was obtained after cytapheresis at the Etablissement Français du Sang Centre-Atlantique (Tours, France). Peripheral blood mononuclear cells (PBMC) were purified on a Ficoll gradient (d=1.077). PBMC were then washed twice at 600g and 400g before being placed in flasks (2.10^8 cells in 175cm²) in RPMI1640 + 5% FCS for 45 min at 37°C + 5%

CO_2 to allow monocyte adhesion. Non-adherent cells were removed and conserved for later isolation of $CD4^+$ $CD25^-$ T lymphocytes. Monocytes were cultured in RPMI1640 + 10% FCS + 1% L-Glutamin + 1% Penicillin/Streptomycin (complete medium) supplemented with 500IU/mL rhIL-4 and 1000IU/mL rhGM-CSF for five days at 37°C + 5% CO_2 in order to obtain immature dendritic cells (DCs). At day 5, DCs were harvested, enumerated in Trypan blue and cultured in 6-well plates at 10^6 DCs/mL in complete medium + rhIL-4 + rhGM-CSF in the presence of LPS (50ng/mL) or BbC50sn (100µg/mL) for two days. Mature DCs (mDCs) were then collected, enumerated and analysed by flow cytometry. LPS-DCs were frozen at -80°C until use in the T cell functional assays; BbC50sn-DCs were either cultured with $CD4^+$ $CD25^-$ T lymphocytes or frozen at -80°C and thawed for repeated stimulation of T lymphocytes.

Long-term co-culture of BbC50sn-DCs and $CD4^+$ $CD25^-$ lymphocytes

$CD4^+$ T lymphocytes were isolated from Peripheral Blood Lymphocytes (PBL) with the Dynal CD4 positive isolation kit (Invitrogen, France) according to the manufacturer's instructions. $CD4^+$ $CD25^+$ T cells were then removed with an anti-CD25 antibody (clone M-A251, BD Biosciences) and anti-mouse IgG magnetic beads (CELLection™ Pan Mouse IgG Kit, Dynal). Isolated $CD4^+$ $CD25^-$ T cells were subsequently enumerated in Trypan blue and either cultured with BbC50sn-DCs or frozen at -80°C until functional assays. Allogeneic $CD4^+$ $CD25^-$ T lymphocytes (5 x 10^5/mL) were cultured with BbC50sn-DCs (1.5 x 10^5/mL) in a 12-well plate in complete RPMI1640 medium. $CD4^+$ $CD25^-$ T lymphocytes were then re-stimulated with previously frozen BbC50sn-DCs once a week for 3 weeks. From the first re-stimulation (day 7 of co-culture), 2IU/mL rhIL-2 and 500IU/mL rhIL-4 were added to the culture medium. Half the medium was replaced between each re-stimulation when necessary. At the end of co-culture (day 28), cells were harvested, and $CD4^+$ T cells were isolated with the Dynal CD4 positive isolation kit (Invitrogen, France). BbC50sn-T cells were enumerated, analysed by flow cytometry and assayed in functional tests. Co-culture supernatants were stored at -20°C until analysis by ELISA.

Functional assays of BbC50sn- T cells

Long term co-cultured T cells (BbC50sn-T cells) were tested for their ability to inhibit proliferation of syngeneic or third party donor $CD4^+$ $CD25^-$ T cells (responder cells) induced by allogeneic or third party LPS-DCs (mDCs): 3 x 10^4 DCs and 10^5 $CD4^+$ $CD25^-$ T cells / well were placed in a V-bottomed 96-well plate. BbC50sn-T cells were irradiated at 16 Gy or were left un-irradiated and added to the plate at several ratios for 5 days. For some experiments, $CD4^+$ $CD25^-$ T cells (R) were treated with mitomycin (50µg/ml) or activated with anti-CD3/CD28 beads (Dynal, cell:bead ratio = 1:1). When specified, BbC50sn-T cells were placed in the upper chamber of Inserts (Nunc 0.2 µm). To test IL-10 and TGF-β involvement in suppression, neutralizing anti-IL-10, -IL-10R and -TGF-β antibodies (R&D systems) or their corresponding isotype controls were added to the medium (at 20 µg/mL, 5 µg/mL, and 20 µg/ml, respectively). Proliferation was then assessed after ^{3}H-Thymidine (0.5 µCi) incorporation during the last 18 h of co-culture. Cells were harvested on a 96-Unifilter plate (Perkin Elmer, France) and ^{3}H-Thymidine incorporation was measured using a liquid scintillation counter (Perkin Elmer). Results are expressed in counts per minute (cpm) ± SEM. Functional assay supernatants were stored at -20°C until analysis by ELISA.

Cell analysis by flow cytometry

BbC50sn-T cells were resuspended in PBS + 5% SVF + 0.1% NaN_3 for staining and several fluo-conjugated antibodies were added at saturated concentrations for 30 min at 4°C (antibodies used are described above). Cells were then washed twice and at least 20,000/sample were analysed using a BD FACSCanto™ cytometer (BD Biosciences). Analysis was performed with BD FACSDiva™ software.

Analysis of cytokine production in culture supernatants

Culture supernatants from long-term BbC50sn-T cells and functional assays were stored at -20°C until analysis. IL-10, IFN-γ and TGF-β production was determined by ELISA with the Ready-Set-Go kit (eBioscience) according to the manufacturer's instructions.

Statistical analysis

Data are presented as means ± SEM. Analysis was performed using the GraphPad Prism 3.0 software, and all data were normally distributed. Comparisons between groups were performed by Student's unpaired or paired 2-tailed *t*-test. Statistical significance was set at $p<0.05$ (*), $p<0.01$(**), $p<0.001$ (***).

RESULTS

BbC50sn-T cells have characteristics of regulatory T cells

BbC50sn-T cells were obtained after repeated stimulation of $CD4^+$ $CD25^-$ T cells with allogeneic BbC50sn-DCs once a week for four weeks. At the end of long-term co-culture, BbC50sn-T cells showed weak proliferation when activated by LPS-

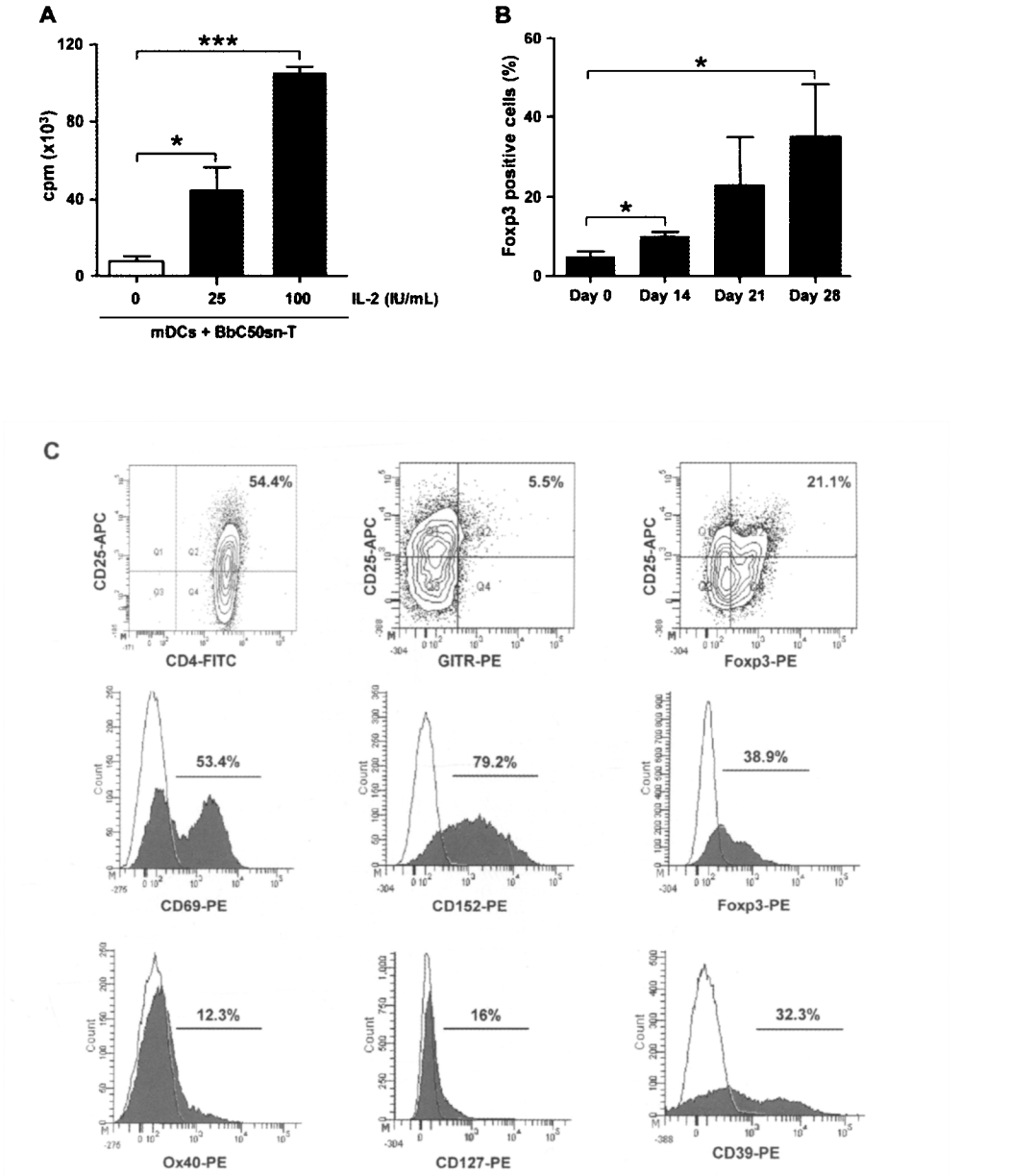

Fig. 1. *BbC50sn-T cells have characteristics of regulatory T cells. The proliferation of BbC50sn-T cells stimulated by mDCs with or without IL-2 was analysed by ^{3}H-Thymidine incorporation (**A**), and cells stained with monoclonal antibodies or their isotype control were analysed by flow cytometry (**B, C**). **A, C**) Results represent one out of 10 independent experiments. **B**) Results represent mean ± SEM of 3 independent experiments. (*) $p<0.05$, (***) $p<0.001$.*

DCs (Fig. 1A). IL-2 treatment restored BbC50sn-T cell proliferation in a dose-dependent manner (Fig. 1A). Flow cytometry was then used to analyse the BbC50sn-T cell phenotype. Results presented in Fig. 1B showed that the percentage of Foxp3-positive cells increased each week following the first re-stimulation. At the end of long-term co-culture, about 54% of BbC50sn-T cells were positive for CD25 and CD69, 32.3% were CD39^{+}, 16% were CD127^{+} and about 80% expressed intracellular CTLA-4 (CD152), whereas expression of GITR and Ox40 was weak (Fig. 1C). Moreover, about 39% of

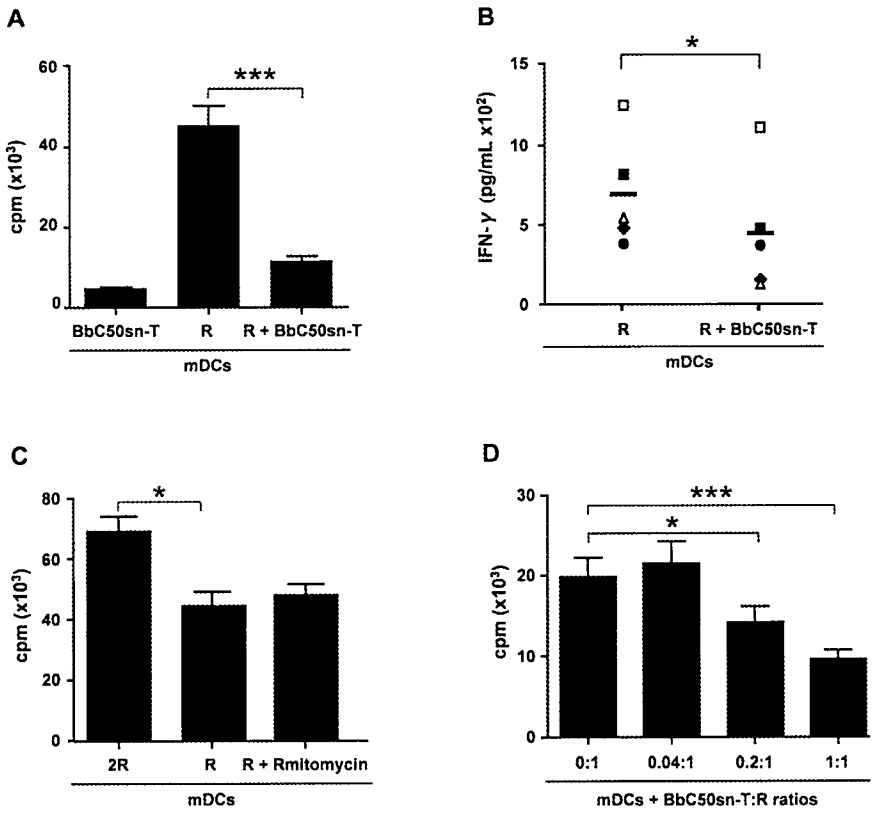

Fig. 2. *BbC50sn-T cells exert a suppressive activity in an MLR. CD4⁺ CD25⁻ T cells treated with mitomycin (Rmitomycin) or left untreated (R) were co-cultured with allogeneic mDCs for 5 days with or without BbC50sn-T cells at a 1:1 ratio (**A, B, C**) or at different BbC50sn-T:R ratios (**D**). T-cell proliferation was analysed by ^{3}H-thymidine incorporation; results are expressed in cpm (triplicate mean ± SEM) (A, C, D). Data represent one out of 12 (A) or 3 (C, D) experiments. B) MLR supernatants were collected before ^{3}H-Thymidine addition and ELISA assessed IFN-γ production. Data represent results of 5 independent experiments. (*) $p<0.05$, (***) $p<0.001$.*

BbC50sn-T cells were positive for Foxp3 expression, 21% of which also expressed CD25.

BbC50sn-T cells inhibit CD4⁺ CD25⁻ T cell proliferation and IFN-γ production in a MLR

BbC50sn-T cells were further examined for their ability to inhibit T cell proliferation and IFN-γ production in a MLR. Allogeneic mDCs were used to activate CD4⁺ CD25⁻ responder T cells (R). BbC50sn-T cells, obtained from the same donor as responder T cells, were then added to this MLR at a ratio of 1:1 for five days, and lymphocyte proliferation was assessed by ^{3}H-Thymidine incorporation. As shown in Fig. 2, A and B, responder T cell proliferation and responder T cell IFN-γ production were significantly decreased in the presence of BbC50sn-T cells. The lower lymphocyte proliferation was not due to exhaustion of the culture medium because the proliferation level of the responder T cells did not decrease after addition of the same number of CD4⁺ CD25⁻ responder T cells, whether treated with mitomycin or not (Fig. 2C). Moreover, the inhibition of responder T cell proliferation was dose dependent (Fig. 2D). Taken together, these results show that BbC50sn-DCs can generate functional allogeneic regulatory T cells.

BbC50sn-T cells exert bystander suppressive activity after antigen-specific activation

In the previous experiments, the CD4⁺ CD25⁻ responder T cells used in functional tests and BbC50sn-T cells came from the same donor. In the experiments described here, responder T cells came from a third party donor and were called "third party

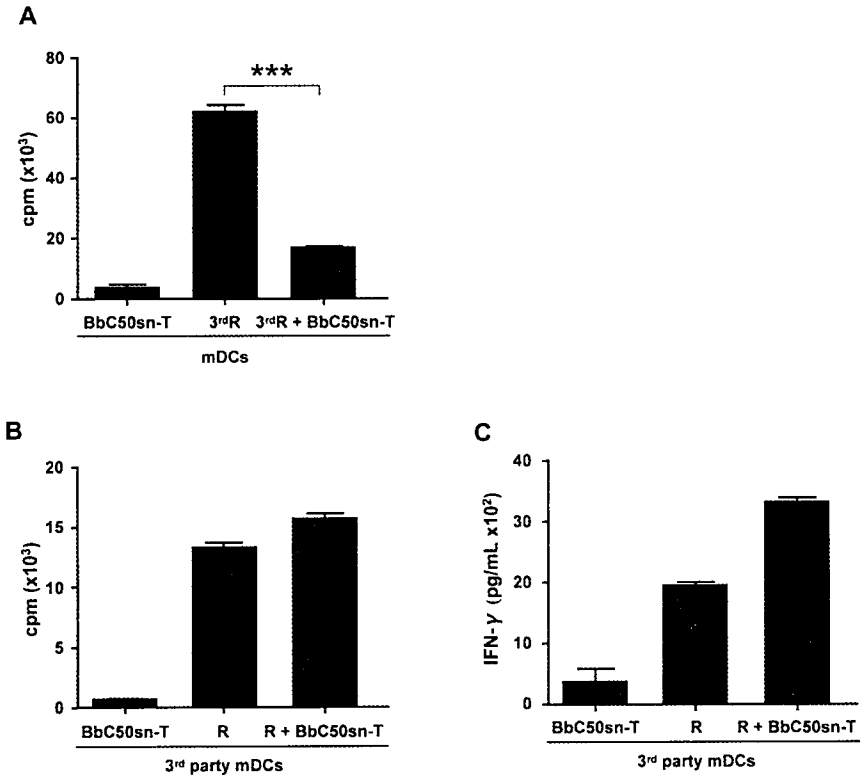

Fig. 3. *BbC50sn-T cells exert a bystander suppressive activity after antigen-specific activation. A) Third party donor CD4⁺ CD25⁻ responder T cells (3rdR) were cultured with allogeneic mDCs with or without BbC50sn-T cells for 5 days. B, C) CD4⁺ CD25⁻ responder T cells (R) were cultured with third party donor mDCs (3rd party mDCs) with or without BbC50sn-T cells for 5 days. T-cell proliferation was analysed by ³H-thymidine incorporation; results are expressed in cpm (triplicate mean ± SEM) (A, B). Co-culture supernatants were collected and IFN-γ production was measured by ELISA (C). Data are representative of one out of 5 (A, B) or 3 (C) experiments. (***) p<0.001.*

responder T cells" (3rd R). The results presented in Fig. 3A show that BbC50sn-T cells still inhibited responder T cell proliferation, although the two populations were not from the same donor.

The regulatory activity of BbC50sn-T cells was also tested in the presence of third party mDCs in order to test whether an alloantigen-specific stimulation was necessary. Third party mDCs came from a third donor, different from the donors of BbC50sn-T cells and of responder T cells. As with allogeneic mDCs, BbC50sn-T cells again showed weak proliferation (Fig. 3B). Nevertheless, BbC50sn-T cells did not suppress CD4⁺ CD25⁻ T cell proliferation when third party mDCs were used (Fig. 3B). Moreover, BbC50sn-T cells did not inhibit IFN-γ production by responder T cells in the presence of the third party mDCs (Fig. 3C). Bystander suppression of BbC50sn-T cells was therefore only effective after activation by mDCs in an antigen-specific manner.

Suppressive activity of BbC50sn-T cells is not mediated by IL-10 or TGF-β

Transwell experiments were carried out in order to determine whether the suppressive activity of BbC50sn-T cells was cell contact-dependent. No suppressive activity was observed when BbC50sn-T cells were placed alone in the upper chamber of the Transwell (Fig. 4A). However, the suppressive activity of BbC50sn-T cells was restored when BbC50sn-T cells were in contact with mDCs in the upper chamber of the Transwell. These results showed that BbC50sn-T cells did not exert their suppressive activity through a T cell – T cell contact but required the presence of mDCs to inhibit a MLR.

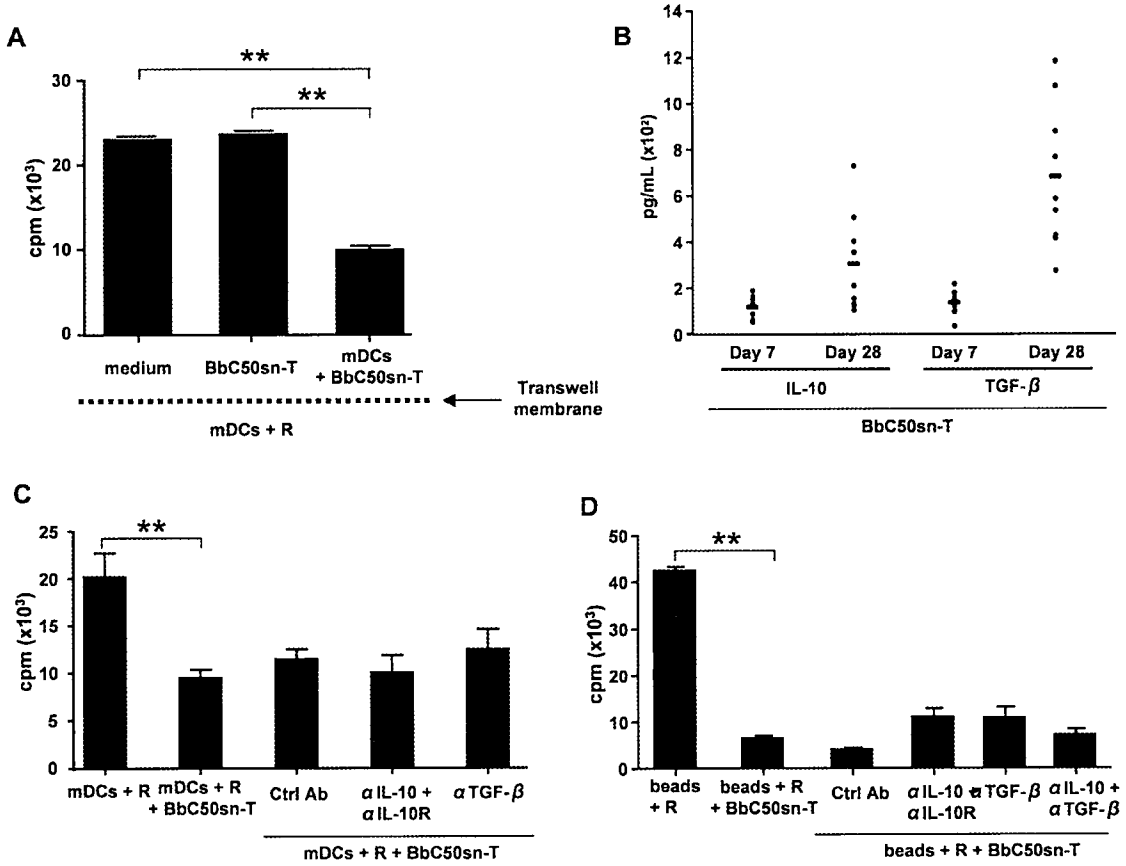

Fig. 4. *Suppressive activity of BbC50sn-T cells is not mediated by IL-10 or TGF-β. A) CD4⁺ CD25⁻ responder T cells (R) were cultured with allogeneic mDCs in the lower chambers of Transwells for 5 days. When specified, BbC50sn-T cells were added to the upper chambers of Transwells with or without mDCs. B) IL-10 and TGF-β production in long-term culture supernatants was assessed by ELISA at days 7 and 28. C) CD4⁺ CD25⁻ responder T cells (R) were cultured with allogeneic mDCs or (D) anti-CD3/CD28 beads in the presence of BbC50sn-T cells with or without anti-IL-10, IL-10R, and/or -TGF-β antibodies or their isotype controls. D) BbC50sn-T cells were placed in the upper chambers of culture inserts. T-cell proliferation was analysed by ³H-thymidine incorporation; results are expressed in cpm (triplicate mean ± SEM). Data are representative of one out of: 5 (A), 4 (C) or 2 (D) independent experiments. B) shows 9 independent experiments with media value represented by a black line. (**) p<0.01.*

Analysis of the supernatants collected during the long-term co-culture showed that IL-10 and TGF-β concentrations increased respectively two-fold and four-fold between day 7 and day 28. At the end of long-term co-culture, BbC50sn-T cells produced about 330pg/mL IL-10 and 700pg/mL TGF-β (Fig. 4B). $CD4^+$ $CD25^-$ lymphocyte proliferation was measured in the presence of anti-IL-10, IL-10R, and/or -TGF-β monoclonal antibodies and their respective isotype controls in an MLR. Responder T cells were activated by mDCs (Fig. 4C) or anti-CD3/CD28 beads (Fig. 4D). As shown in Fig. 4, responder T-cell proliferation inhibited by BbC50sn-T cells was not restored when anti-IL-10, IL-10R, and/or -TGFβ antibodies were added to the MLR. IL10 and TGFβ therefore did not appear to mediate the suppressive activity of BbC50sn-T cells.

DISCUSSION

Dendritic cells have a key role in inducing both effector and regulatory immune responses (1). We have previously shown that the TLR2 maturation of BbC50sn-DCs conferred on them a pro-tolerogenic

profile (26). In the present study, we demonstrated that BbC50sn-DCs induced functional allogeneic regulatory T cells *ex vivo*. BbC50sn-T cells were anergic, secreted significant amounts of IL-10 and TGF-β, inhibited alloantigen-driven proliferation of $CD4^+$ $CD25^-$ T cells in a dose-dependent manner and suppressed IFN-γ production of $CD4^+$ $CD25^-$ responder T cells. These features are specific to regulatory T cells (27).

Several studies have reported a modulatory effect of probiotics on DCs (15, 16, 26). Only a few have studied the direct action of such DCs on regulatory T-cell induction in mouse models as well as in humans (28, 29). Nevertheless, the possibility of regulatory T-cell expansion cannot be excluded in most of these studies concerning probiotic bacteria. As the $CD4^+$ T cells used in the present study were depleted of $CD4^+$ $CD25^+$ natural regulatory T cells before the long-term co-culture, the BbC50sn-T cells obtained were therefore *de novo*-induced regulatory T cells. Thus, this is, to the best of our knowledge, the first report of *in vitro* induction of human allogeneic regulatory T cells by DCs treated with a bacteria-free fermentation product obtained with a probiotic strain.

The modulatory capacity of BbC50sn on DCs is strengthened since the allogeneic response represents a strong Th1 response. Furthermore, our results support the concept that mature DCs can induce regulatory T cells (10) even though not every type of mature DC is able to induce regulatory T cells. Indeed, we previously demonstrated with the same *ex vivo* culture model that TNFα-treated DCs did not induce regulatory T cells (30).

BbC50sn-T cells were hyporesponsive to further stimulation by mDCs but IL-2 restored BbC50sn-T-cell proliferation in a dose-dependent manner; BbC50sn-DCs therefore induced anergy of $CD4^+$ T cells *ex vivo* (31). Analysis of BbC50sn-T cells by flow cytometry revealed that this T-cell population was heterogeneous and expressed CD25, CTLA-4, CD69, CD39, CD127 and Foxp3 at several levels. These molecules are usually expressed by regulatory T cells but are not strictly specific to regulatory T cells. Foxp3 expression is directly correlated with the regulatory activity of T cells in mice, but not in humans (27). About 39% of BbC50sn-T cells expressed Foxp3 in this study. However, we do not know whether the whole suppressive activity of BbC50sn-T cells described here was only due to these $Foxp3^+$ T cells.

Transwell experiments showed that BbC50sn-T cells did not inhibit responder T-cell proliferation when they were not in contact with mDCs. Moreover, they did not inhibit responder T-cell proliferation or IFN-γ production when they were stimulated by third party mDCs. BbC50sn-T cells thus required direct contact with alloantigen-specific mDCs in order to exert their suppressive activity. However, although the activation of regulatory T cells was Ag-specific, the suppressive effect was Ag-independent. This regulatory mechanism has already been described as "bystander suppression" (32). "Bystander suppression" is defined as a process whereby antigen-specific adaptive regulatory T cells inhibit the T effector-cell response both to specific antigen and to a third-party antigen. Interestingly, this expression has also been used in the field of oral tolerance (32). In the present study, BbC50sn-T cells still inhibited $CD4^+$ $CD25^-$ T-cell proliferation when they were separated from responder T cells by a semi-permeable membrane. The suppressive activity of BbC50sn-T cells was probably mediated by a soluble factor that remains to be identified. BbC50sn-T cells produced IL-10 and TGF-β, both known to be mainly secreted by regulatory T cells (27). Nevertheless, the suppressive activity of BbC50sn-T cells was not mediated by these cytokines. Numerous ways of suppression were evidenced for the different types of regulatory T cells (27). Nitric oxide (NO) has also been shown to mediate regulatory activity (33) but was not responsible for the suppressive activity of BbC50sn-T cells in our model (data not shown). About 32% of BbC50sn-T cells expressed CD39, and therefore it is possible that BbC50sn-T cells exert their suppressive activity *via* this ectonucleotidase (34). Both DCs and regulatory T cells have an important role in intestinal homeostasis and allergy regulation (7). Intestinal homeostasis is in part dependent on tolerance of commensal bacteria by the intestinal immune system as it has been shown that a breakdown in immune tolerance to commensal bacteria leads to the development of inflammatory bowel diseases (2). BbC50sn down-regulated inflammation at the epithelial level *in vitro* and protected mice from TNBS-induced colitis through

its action on murine DCs (25). Our results suggest that the decreased intestinal inflammation obtained via BbC50sn-DCs could be due to the generation of syngeneic regulatory T cells through regulatory DCs.

In summary, we demonstrated in the present study that human DCs treated with a bacteria-free fermentation product could induce *de novo* allogeneic regulatory T cells *ex vivo*. These results contribute to the understanding of the mechanisms of action of probiotic bacteria and may help to explain the health benefits observed when administrating probiotic in human clinical trial (35).

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REFERENCES

1. Steinman RM. Dendritic cells: understanding immunogenicity. Eur J Immunol 2007; 37(S):S53-60.
2. Rescigno M, Di Sabatino A. Dendritic cells in intestinal homeostasis and disease. J Clin Invest 2009; 119:2441-50.
3. Chehade M, Mayer L. Oral tolerance and its relation to food hypersensitivities. J Allergy Clin Immunol 2005; 115:3-12.
4. Westerberg LS, Klein C, Snapper SB. Breakdown of T-cell tolerance and autoimmunity in primary immunodeficiency-lessons learned from monogenic disorders in mice and men. Curr Opin Immunol 2008; 20:646-54.
5. Akdis M, Verhagen J, Taylor A, et al. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. J Exp Med 2004; 199:1567-75.
6. Sakaguchi S, Wing K, Miyara M. Regulatory T cells - a brief history and perspective. Eur J Immunol 2007; 37(S):S116-23.
7. Barnes MJ, Powrie F. Regulatory T cells reinforce intestinal homeostasis. Immunity 2009; 31:401-11.
8. Jonuleit H, Schmitt E, Schuler G, Knop J, Enk AH. Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. J Exp Med 2000; 192:1213-22.
9. Levings MK, Gregori S, Tresoldi E, Cazzaniga S, Bonini C, Roncarolo MG. Differentiation of Tr1 cells by immature dendritic cells requires IL-10 but not CD25+CD4+ Tr cells. Blood 2005; 105:1162-69.
10. Baba N, Samson S, Bourdet-Sicard R, Rubio M, Sarfati M. Commensal bacteria trigger a full dendritic cell maturation program that promotes the expansion of non-Tr1 suppressor T cells. J Leukoc Biol 2008; 84:468-76.
11. Rembacken BJ, Snelling AM, Hawkey PM, Chalmers DM, Axon AT. Non-pathogenic Escherichia coli versus mesalazine for the treatment of ulcerative colitis: a randomised trial. Lancet 1999; 354:635-39.
12. Shanahan F. Irritable bowel syndrome: shifting the focus toward the gut microbiota. Gastroenterology 2007; 133:340-42.
13. Feleszko W, Jaworska J, Rha R-D, et al. Probiotic-induced suppression of allergic sensitization and airway inflammation is associated with an increase of T regulatory-dependent mechanisms in a murine model of asthma. Clin Exp Allergy 2007; 37:498-505.
14. Wolvers D, Antoine J-M, Myllyluoma E, Schrezenmeir J, Szajewska H, Rijkers GT. Guidance for substantiating the evidence for beneficial effects of probiotics: prevention and management of infections by probiotics. J Nutr 2010; 140:698S-712S.
15. Foligne B, Zoumpopoulou G, Dewulf J, et al. A key role of dendritic cells in probiotic functionality. PLoS ONE 2007; 2:e313.
16. Hart AL, Lammers K, Brigidi P, et al. Modulation of human dendritic cell phenotype and function by probiotic bacteria. Gut 2004; 53:1602-9.
17. de Roock S, van Elk M, van Dijk MEA, et al. Lactic acid bacteria differ in their ability to induce functional regulatory T cells in humans. Clin Exp Allergy 2010; 40:103-10.
18. Adams CA. The probiotic paradox: live and dead cells are biological response modifiers. Nutr Res Rev

2010; 23:37-46.
19. Vinderola G, Perdigón G, Duarte J, Farnworth E, Matar C. Effects of the oral administration of the exopolysaccharide produced by *Lactobacillus kefiranofaciens* on the gut mucosal immunity. Cytokine 2006; 36:254-60.
20. Vinderola G, Matar C, Palacios J, Perdigón G. Mucosal immunomodulation by the non-bacterial fraction of milk fermented by *Lactobacillus helveticus* R389. Int J Food Microbiol 2007; 115:180-86.
21. Pochard P, Hammad H, Ratajczak C, Charbonnier-Hatzfeld A-S, Just N, Tonnel A-B, Pestel J. Direct regulatory immune activity of lactic acid bacteria on Der p 1-pulsed dendritic cells from allergic patients. J Allergy Clin Immunol 2005; 116:198-204.
22. Gerbitz A, Schultz M, Wilke A, Linde H-J, Schölmerich J, Andreesen R, Holler E. Probiotic effects on experimental graft-versus-host disease: let them eat yogurt. Blood 2004; 103:4365-67.
23. Agrewala JN, Suvas S, Singh V, Vohra H. Delivery of antigen in allogeneic cells preferentially generates $CD4^+$ Th1 cells. Clin Exp Immunol 2003; 134:13-22.
24. Obata F, Yoshida K, Ohkubo M, et al. Contribution of CD4+ and CD8+ T cells and interferon-gamma to the progress of chronic rejection of kidney allografts: the Th1 response mediates both acute and chronic rejection. Transpl Immunol 2005; 14:21-25.
25. Heuvelin E, Lebreton C, Grangette C, Pot B, Cerf-Bensussan N, Heyman M. Mechanisms involved in alleviation of intestinal inflammation by bifidobacterium breve soluble factors. PLoS ONE 2009; 4:e5184.
26. Hoarau C, Lagaraine C, Martin L, Velge-Roussel F, Lebranchu Y. Supernatant of *Bifidobacterium breve* induces dendritic cell maturation, activation, and survival through a Toll-like receptor 2 pathway. J Allergy Clin Immunol 2006; 117:696-702.
27. Shevach EM. From vanilla to 28 flavors: multiple varieties of T regulatory cells. Immunity 2006; 25:195-201.
28. Jeon SG, Kayama H, Ueda Y, et al. Probiotic *Bifidobacterium breve* induces IL-10-producing Tr1 cells in the colon. PLoS Pathog 2012; 8:e1002714.
29. Konieczna P, Groeger D, Ziegler M, et al. *Bifidobacterium infantis* 35624 administration induces Foxp3 T regulatory cells in human peripheral blood: potential role for myeloid and plasmacytoid dendritic cells. Gut 2012; 61:354-66.
30. Lagaraine C, Lemoine R, Baron C, Nivet H, Velge-Roussel F, Lebranchu Y. Induction of human CD4+ regulatory T cells by mycophenolic acid-treated dendritic cells. J Leukoc Biol 2008; 84:1057-64.
31. Schwartz RH. T cell anergy. Annu Rev Immunol 2003; 21:305-34.
32. Miller A, Lider O, Weiner HL. Antigen-driven bystander suppression after oral administration of antigens. J Exp Med 1991; 174:791-98.
33. Chen C, Lee W, Zhong L, Liu C-P. Regulatory T cells can mediate their function through the stimulation of APCs to produce immunosuppressive nitric oxide. J Immunol 2006; 176:3449-60.
34. Hilchey SP, Kobie JJ, Cochran MR, et al. Human follicular lymphoma CD39+-infiltrating T cells contribute to adenosine-mediated T cell hyporesponsiveness. J Immunol 2009; 183:6157-66.
35. Morisset M, Aubert-Jacquin C, Soulaines P, Moneret-Vautrin D-A, Dupont C. A non-hydrolyzed, fermented milk formula reduces digestive and respiratory events in infants at high risk of allergy. Eur J Clin Nutr 2011; 65:175-83.