DOI 10.1002/mnfr.201300596 *Mol. Nutr. Food Res.* 2014, *58*, 1132–1143

RESEARCH ARTICLE

Altered human gut dendritic cell properties in ulcerative colitis are reversed by *Lactobacillus plantarum* extracellular encrypted peptide STp

Hafid O. Al-Hassi[1], Elizabeth R. Mann[1*], Borja Sanchez[2], Nicholas R. English[1], Simon T.C. Peake[1,3], Jonathan Landy[1,3], Ripple Man[3], Maria Urdaci[4], Ailsa L. Hart[3], Luis Fernandez-Salazar[5], Gui Han Lee[1,3], Jose A. Garrote[6], Eduardo Arranz[7], Abelardo Margolles[8], Andrew J. Stagg[9], Stella C. Knight[1] and David Bernardo[1]*

[1] Antigen Presentation Research Group, Imperial College London, Northwick Park and St. Mark's Campus, Harrow, UK
[2] Nutrition and Bromatology Group, Department of Analytical and Food Chemistry, Food Science and Technology Faculty, University of Vigo, Ourense Campus, Ourense, Spain
[3] St. Mark's Hospital, North West London Hospitals NHS Trust, Harrow, UK
[4] Laboratoire de Microbiologie et Biochimie Appliquee, Ecole Nationale Superieure des Sciences Agronomiques de Bordeaux, Gradignan, France
[5] Gastroenterology Service, Hospital Clinico Universitario de Valladolid, Valladolid, Spain
[6] Department of Genetics and Molecular Biology, Clinical Laboratory Service, Hospital Universitario Rio Hortega, Valladolid, Spain
[7] Department of Paediatrics and Immunology, Mucosal Immunology Service, Universidad de Valladolid, IBGM-CSIC, Valladolid, Spain
[8] Departmento de Microbiologia y Bioquimica de Productos Lacteos, Instituto de Productos Lacteos de Asturias, Consejo Superior de Investigaciones Cientificas, Villaviciosa, Spain
[9] Centre for Immunology and Infectious Disease, Barts and the London School of Medicine and Dentistry, Blizard Institute of Cell and Molecular Science, Queen Mary University of London, UK

Received: August 14, 2013
Revised: October 9, 2013
Accepted: October 18, 2013

Scope: The human/microbiota cross-talk is partially mediated by bacteria-derived peptides like Serine-Threonine peptide (STp), which is resistant to gut proteolysis, is found in the human healthy colon and induces regulatory properties on gut dendritic cells (DCs); here we characterized human gut DC in ulcerative colitis (UC) patients and studied the effect of STp on their properties.

Methods and results: Human colonic DC from healthy controls and UC patients were isolated, conditioned for 24 h +/− STp and characterized by flow cytometry, immunohistochemistry, and electron microscopy. Expression of immature DC markers DC-SIGN and ILT3, and Toll-like receptors were increased on gut UC-DC. Langerin (involved in phagocytosis), lymph node homing marker CCR7, and activation markers CD40/CD80/CD86 were decreased in UC. Gut DC had restricted stimulatory capacity for T-cells in UC. Conditioning of DC with STp in vitro reduced Toll-like receptor expression, increased CD40 and CD80 expression, and restored their stimulatory capacity.

Conclusion: Colonic DCs display an abnormal immature phenotype in UC, which was partially restored following STp treatment. Bacteria-derived metabolites, like STp, seem to have a role in gut homeostasis that is missing in UC so they might lead a new era of probiotic products setting the basis for nondrug dietary therapy in inflammatory bowel disease.

Correspondence: Professor Stella C. Knight, Antigen Presentation Research Group, Imperial College London, Northwick Park and St. Mark's Campus, Level 7W St. Mark's Hospital, Watford Road, Harrow, HA1 3UJ, UK
E-mail: s.knight@imperial.ac.uk
Fax: +44-0-20-8869-3532

Abbreviations: **CCR**, C-chemokine receptor; **DC**, dendritic cells; **EM**, electron microscopy; **IBD**, inflammatory bowel disease; **IR**, intensity ratio; **LP**, lamina propria; **MLR**, mixed leukocyte reaction; **STp**, serine-threonine peptide; **TLRs**, Toll-like receptors; **UC**, ulcerative colitis; **UCDAI**, UC disease activity index

*These authors have contributed equally to this work.

Keywords:
Dendritic Cells / Microbiota / Postbiotics / STp / Ulcerative colitis

1 Introduction

Ulcerative colitis (UC) results from inappropriate mucosal immune responses to constituents of the intestinal flora in genetically predisposed individuals [1]. Antigen-presenting cells such as dendritic cells (DCs) are likely to play a central role in the host response to intestinal flora, both in innate responses to bacteria and by shaping the properties of adaptive immune responses [2]. Indeed, DCs are key initiators of innate immune responses and the only cells capable of generating primary T-cell responses [3].

Intestinal DCs are present within Peyer's patches and distributed throughout the lamina propria (LP), and maintain the delicate balance in the gut between immunogenicity against invading pathogens and tolerance of the commensal microbiota. Disruption of this balance can result in inflammatory bowel disease (IBD) [4, 5]. In health, homeostatic effects of commensal microenvironment are not, therefore, merely providing a "tolerogenic" milieu but contribute a balance between pro-inflammatory and anti-inflammatory responses. Responses of DC to microbial antigens are enabled partly through their rich supply of pattern recognition receptors such as Toll-like receptors (TLRs), which recognize structural elements on bacterial cell surfaces [6]. Intestinal immune tolerance is initiated by immature DCs that induce anergic T-cells [7]. Upon DC activation lymph node homing marker C-chemokine receptor 7 (CCR7) is upregulated on DC enabling migration to secondary lymphoid tissue in order to generate primary T-cell responses [8]. These T-cell responses generated can be immunogenic or tolerogenic [3, 9], but properties of healthy gut DCs suggest that in the steady state, gut DCs have a homeostatic role, likely to be due to the high antigenic load including the gut microbiota and food antigens. Such DC properties include hyporesponsiveness to LPS stimulation [10], high constitutive production of regulatory cytokine IL-10 and low production of inflammatory IL-12, low expression of costimulatory molecules and TLRs [11], and a restricted capacity to stimulate T-cells [12]. Altered DC phenotype and function in UC suggests that they contribute to UC pathogenesis [5, 13, 14].

The intestinal microbiota is altered in patients with active UC compared with that of healthy individuals [15, 16]. Manipulating gut flora using probiotics may correct and/or prevent dysregulated immune function in UC; the effects of probiotic bacteria on DC, which are so pivotal in early bacterial recognition and shaping T-cell responses are likely to be central in immunomodulation by these bacteria. The probiotic mixture VSL#3 was effective in the treatment of patients with mild to moderately active UC, with a response rate of 50–70% [17] and in animal models VSL#3 altered the phenotype and the distribution of DC in the gut mucosa in vivo [18]. Furthermore, oral administration of VSL#3 in UC patients had direct regulatory effects on human gut DC in vivo that were likely to contribute to therapeutic benefit [19].

There is growing interest in the immunomodulatory effects of products produced by probiotic bacteria in the gut, as well as direct effects of bacteria themselves, partly due to recent clinical studies that have concluded caution needs to be taken when administering live bacteria in patients with acute inflammation [20]. Specific strains of probiotic bacteria can have inflammatory activities in vitro both in healthy and IBD human tissue, but metabolic products of the same bacterial strains can have regulatory effects and protect against inflammatory properties of pathogenic bacteria [21]. We recently identified a peptide (serine-threonine peptide, STp) secreted by *Lactobacillus plantarum* that is resistant to gut proteolysis and present in the colon of the majority of healthy individuals. STp induced regulatory effects on human gut DC in vitro with knock-on effects on the T-cells that they stimulated [22]. These data suggested that the molecular dialog between intestinal bacteria and DCs may be mediated by immunomodulatory peptides secreted by commensal bacteria. In this study, we aimed to investigate this further, in the context of UC. Most studies supporting a role for DC in IBD are based on animal models [23] but we have developed methods for assessing the phenotype and function of human DC, present in small numbers in the gut, from gut biopsies [13].

2 Materials and methods

2.1 Tissues and cells

The study was approved by the Outer West London Regional Ethics Committee and Imperial College London (08/H0717/24). All patients provided written informed consent on entry to the study.

2.1.1 Colonic biopsies

Colonic biopsies were obtained at colonoscopy, following informed consent. Patient groups included UC patients in remission (six female, six male, mean age 48 years), patients with active UC (12 female, 10 male, mean age 52), and healthy controls (13 female, 11 male, mean age 44). Samples were used for different experiments as seen in the figures, so not all the samples were used in all the experiments. Healthy controls had macroscopically and histologically normal intestines, undergoing colonoscopy for rectal bleeding or family history of colorectal cancer. Diagnosis for patients with UC was made using clinical parameters, radiographic studies, endoscopic and histological criteria. Disease activity for UC

was assessed using the UC disease activity index (UCDAI), all active patients had UCDAI > 4, and all patients in remission had UCDAI < 2. Patients were either not receiving therapy, or were on minimal treatment: 5-aminosalicylic acid and/or azathioprine. Biopsies were collected in ice-chilled complete medium.

2.1.2 Intestinal DCs

Total LP cells were obtained from biopsy tissue via collagenase digestion, following removal of mucus and epithelial layers via DTT/EDTA incubation, as previously described [11]. DCs were identified as HLA-DR^+ lineage cocktail$^-$ (HLA-$DR^+CD3^-CD14^-CD16^-CD19^-CD34^-$) by flow cytometry and analyzed for surface phenotype. In some experiments, cells were obtained by a cell migration/"walkout" method during 20-h incubation of biopsies (37°C, 5% CO_2, high humidity) in complete medium (RPMI 1640 (Dutch modification; Sigma-Aldrich, Dorset, UK) supplemented with 10% FCS, 100 μmL^{-1} penicillin, 100 μgmL^{-1} streptomycin and 20 mM L-glutamine after previous DTT/EDTA incubation of freshly obtained biopsies.

2.2 STp conditioning of intestinal DCs

Gut biopsies were incubated for 20 h for the walkout method following DTT/EDTA treatment, in complete medium with or without 1 μg/mL STp, as previously described [22]. Briefly, the gene coding for STp, including the genetic information for a histidine tag, was cloned into pNZ9110 and introduced in *Lactococcus lactis* NZ9000. Gene expression was induced by nisin (40 ng/mL) and STp was purified from the growth supernatant using Ni-NTA agarose (Qiagen Iberia S.L, Madrid, Spain) and proper imidazole buffers for STp washing and elution. Presence of STp was confirmed by Western-blot using a polyclonal serum developed in rabbit. Fractions were extensively dialyzed against PBS and the purified STp identified by N-terminal degradation, preformed in a Procise 494 Protein Sequencer (Applied Biosystems, Foster City, CA) revealing over 99% purity as estimated by SDS-PAGE. STp was subsequently lyophilized and resuspended in complete medium before being added to the cells (negative control included addition of the same volume of complete medium—without STp—to the cells). STp had been cloned and obtained from a Gram-positive bacteria [22], and therefore was LPS-free as also confirmed following the Limulus amebocyte lysate protocol following manufacturer's instructions (Kinetic-QCLTM assay, Lonza, Basel, Switzerland). Following culture of LP cells with and without STp, DCs were identified as HLA-DR^+ lineage cocktail$^-$ by flow cytometry or enriched by centrifugation (600 × *g*, 15 min at RT) over NycoPrep™, and used for T-cell stimulation. These enriched DCs have been characterized as functional colonic DCs [13].

2.3 Enrichment of T-cells

Peripheral blood mononuclear cells were suspended in MiniMACs buffer (PBS containing 0.5% BSA and 2 mM EDTA) and T-cells were enriched by depletion of $CD14^+$, $CD19^+$, and HLA-DR^+ with immunomagnetic beads (Miltenyi Biotech, Bisley, UK) following manufacturer's instructions (over 98% purity of $CD3^+$ T-cells).

2.4 Proliferation assay

Carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen Ltd., UK) labeled T-cells (4×10^5/well) were incubated for 5 days in U-bottomed 96 well microtitre plates with enriched intestinal allogeneic DCs at 3% in a mixed leukocyte reaction (MLR). Cells were recovered and $CFSE^{lo}$ proliferating cells identified and quantified by flow cytometry. T-cells were also cultured in the absence of DCs, which did not proliferate and constituted an internal negative control.

2.5 Antibody labeling

For immunohistochemistry monoclonal antibodies specific for the following markers were used: DC-LAMP/CD208 (104.G4), CD1a (O10), and langerin/CD207 (DCGM4) were from Beckman Coulter High Wycombe, UK. DC-SIGN/CD209 (120507) was from R&D SYSTEMS, Oxford, UK. Avidin biotin reagents, biotinylated goat anti-mouse IgG were from Vector Laboratories, Peterborough, UK.

For flow cytometry, monoclonal antibodies with the following specificities and conjugations were used: CD86-FITC (24F), CD80-FITC (L307.4), CD3-PeCy5 (UCHT1), CCR7 (2H4)-PE, and HLA-DR-APC (G46–6) were purchased from BD Biosciences (Oxford); CD11c-FITC (KB90) was purchased from Dako (Ely, UK); CD40-FITC (LOB7/6), TLR2-FITC (TLR2.3), TLR4-FITC (HTA125), DC exclusion cocktail-PE-Cy5 (CD3 (S4.1), CD14 (TUK4), CD16 (3G8), CD19 (SJ25-C1), CD34 (581) were purchased from AbD Serotec (Oxford); DC-SIGN/CD209-PE (120507) and ILT3-PE (293623) were purchased from R&D systems (Oxford); Langerin/CD207-PE (DCGM4) was purchased from Beckman Coulter (High Wycombe). Appropriate isotype-matched control antibodies were purchased from the same manufacturers. After the staining, cells were fixed with 1% paraformaldehyde in 0.85% saline and stored at 4°C prior to acquisition on the flow cytometer, within 48 h.

2.6 Flow cytometry and data analysis

Data were acquired on a FACSCantoII (BD Biosciences) and analyzed using WinList 5.0 software (Verity, ME).

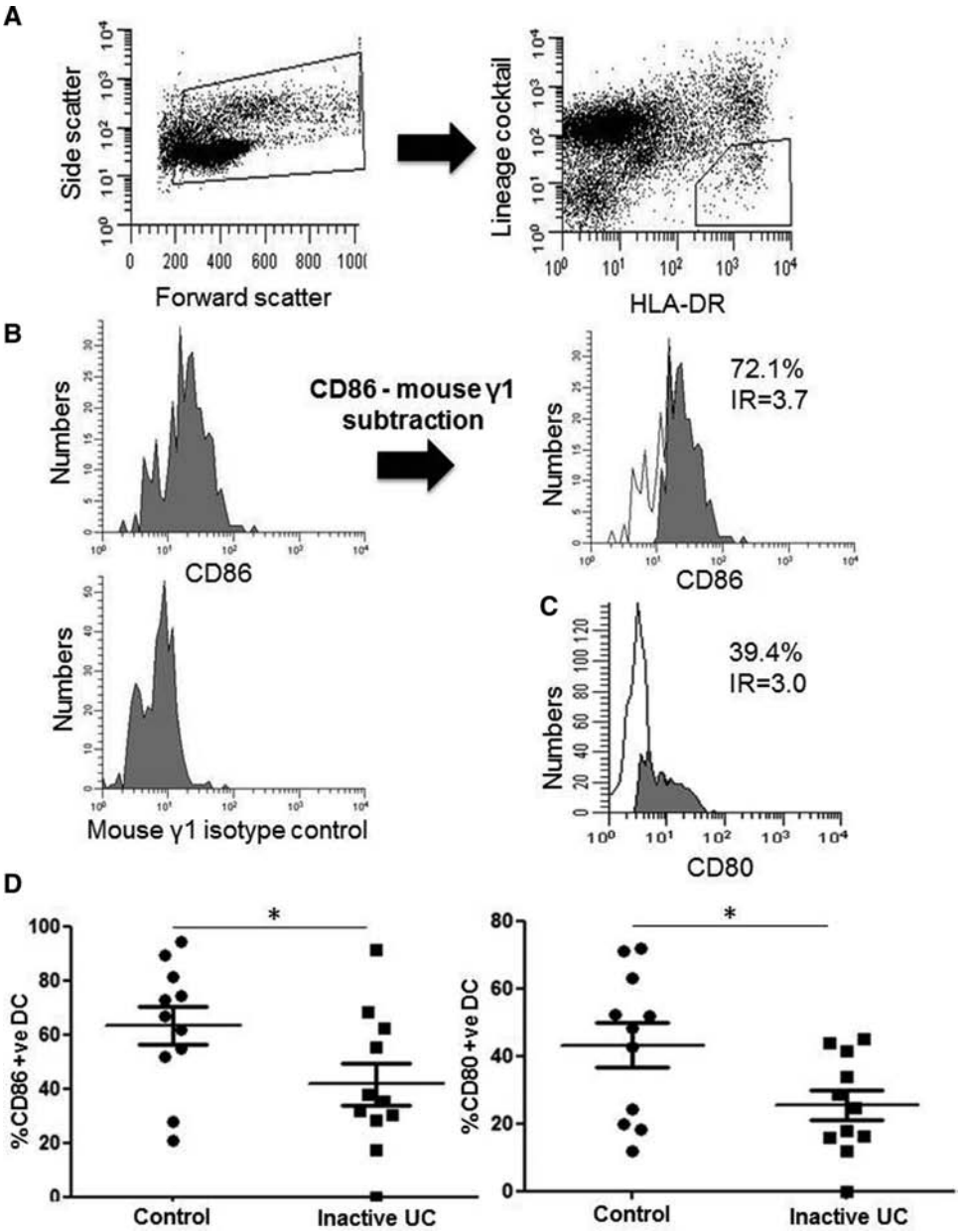

Figure 1. Identification and activation status of DC in biopsies from healthy controls and UC patients. (A) Intestinal LP DCs were identified as HLA-DR^+/lineage $cocktail^-$ cells by multicolour flow cytometry. (B) Isotype control staining histograms were subtracted from the respective test staining for surface markers on DC (CD86 in this example). The shaded areas of histograms represent the fraction of positive cells after subtraction of staining from isotype-matched control (determined both as percentage and as IR of the identified positive cells compared with the isotype labeling) assessed by SED normalized subtraction from the isotype histrogram. (C) Representative histogram for CD80 expression following subtraction from respective isotype (not shown). (D) Expression of the costimulatory molecules CD80 and CD86 on gut DC from inactive UC patients and healthy controls displayed as the mean ± SEM. The statistical significance of differences between groups was determined using unpaired *t*-test and $p < 0.05$ was regarded as significant ($^*p < 0.05$).

2.7 Immunohistochemistry

The avidin-biotin complex technique was employed [24]. Briefly, slides of formalin fixed, paraffin-embedded tissue sections of endoscopic biopsies were deparaffinized and rehydrated and kept moist for the rest of the procedures. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in 100% methanol for 30 min. Tissues were then incubated with normal goat serum for 30 min at room temperature and incubated overnight in a humid atmosphere at 4°C, with primary antibodies; anti-CD1a (1:20); anti-DC-LAMP (1:50); anti-DC-SIGN (1:200), and anti-langerin (1:100). After washing and incubation with biotinylated goat anti-mouse Ig for 30 min, sections were washed and incubated with the avidin-biotin-peroxidase complex. Subsequently, sections were washed in tap water, counterstained with hematoxylin, placed in acid alcohol for 3–5 s and washed in water. Sections were dehydrated, cleared in xylene, and finally mounted with DEPEX. Red-brown staining indicates positive immunoreactivity. Control sections were incubated with normal serum instead of primary antibodies. All incubation times were similar and reactions were stopped simultaneously on sections to standardize the quantitative methods.

For quantification of antibody staining, numbers of positive cells in every possible microscopic field in all sections were counted under high-power magnification (40× objective). To enable comparison between sections of different sizes, results were expressed as number of positive

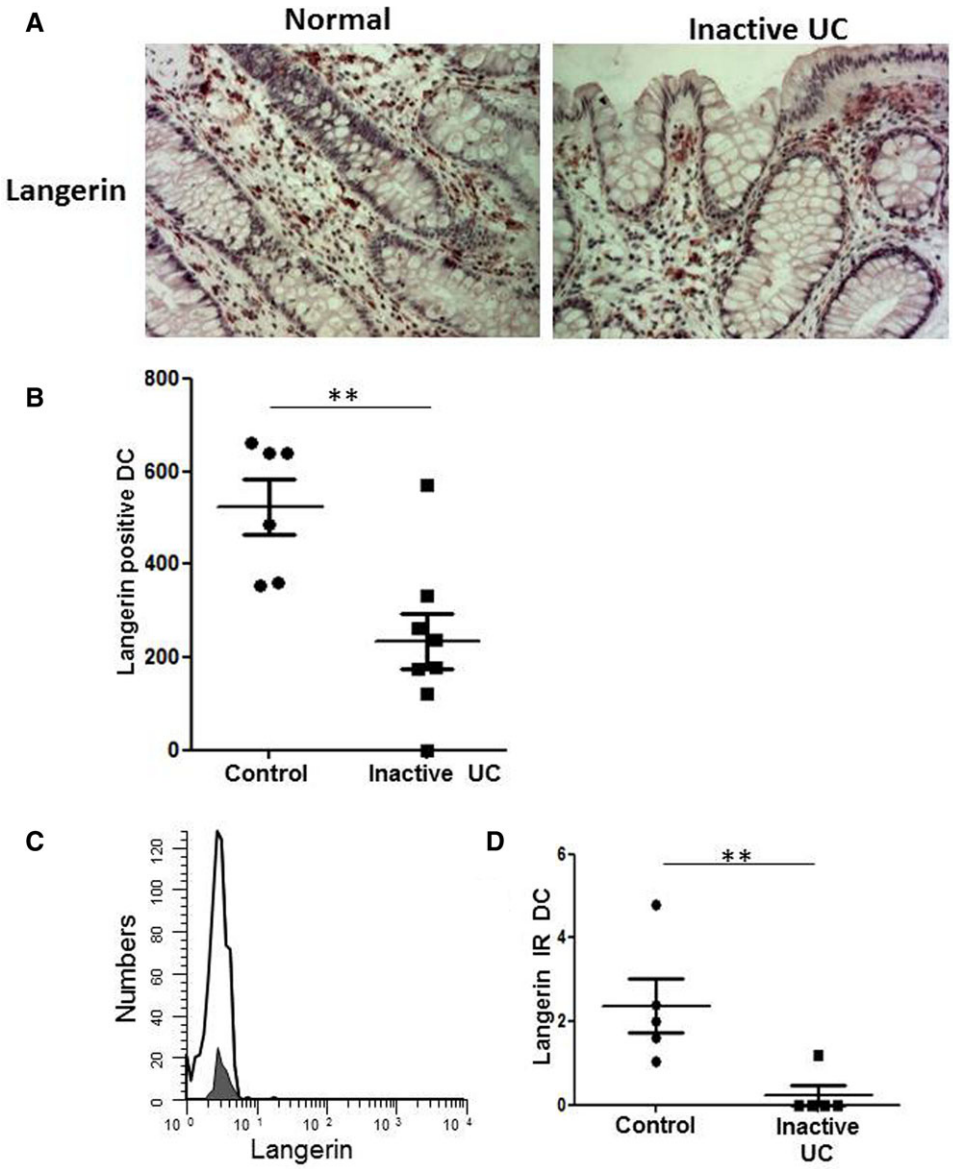

Figure 2. Decreased langerin expression on LP DC from UC patients. (A) Localization of langerin$^+$ DC by immunohistochemistry. In healthy control sections langerin$^+$ immature DCs were scattered throughout the LP while mainly tend to cluster below the epithelium in sections from UC patients. (B) Corresponding average numbers of all langerin$^+$ DC per section (C) Langerin expression (determined as IR) was also determined by flow cytometry as explained in Fig. 1B and (D) data displayed as the mean ± SEM. The statistical significance of differences between groups was determined using unpaired *t*-test and $p < 0.05$ was regarded as significant (**$p < 0.01$).

cells per 10 microscopic fields. Only morphologically well-defined positively stained cells were considered for analysis. Intensity of DC-SIGN immunoreactivity was also quantified using an arbitrary 4-point scale ranging from 0–4 (0 = no staining and 4 = intense staining). All assessments of immunostaining were independently evaluated by two investigators who had no previous knowledge of the clinical data.

2.8 EM

Approximately 2 × 2 mm pieces of tissue were fixed in 0.1 mol/L sodium phosphate buffer, pH 7.4 containing 3% glutaraldehyde for 12 h at 4°C, washed in 0.1M ph 7.4 SPB and post fixed in 1% osmium tetroxide in 0.1% pH 7.4 SPB for 1 h. Samples were then washed and left in distilled water to remove the phosphate for overnight and were block stained in 2% uranyl acetate for 2–4 h. The samples were washed with dH_2O and dehydrated using an acetone gradient and gradually infiltrated with araldite resin. Araldite was changed twice over 4–8 h, then samples were embedded in the araldite resin then cured for 18 h at 65°C. Ultrathin sections (100-nm-thick) were cut on a Reichert-Jung Ultracut E microtome and collected on a 200 mesh copper grid. After staining with Reynold's lead citrate, the grid was carbon coated and visualized using a JEOL 1200 EX electron microscope. DCs were identified as previously described [25–28] and have been classified into three types by electron microscopy (EM): Type 1 DC; small cells with short veils relative to their size, small cytoplasm area, and heterochromatic nuclei with thick chromatin layer surrounding the nucleus and chromatic dense

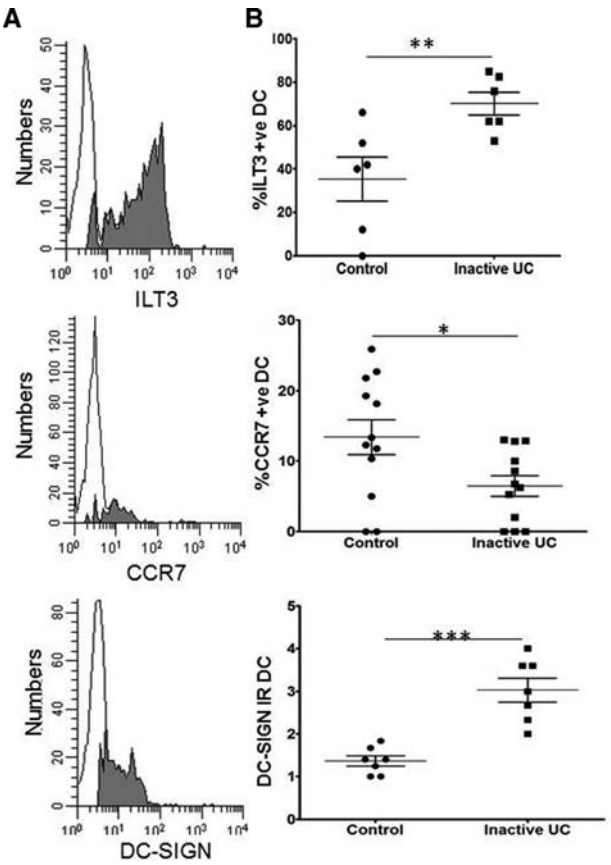

Figure 3. Maturation status of DC in biopsies from healthy controls and UC patients. (A) Expression levels of the inhibitory receptor ILT3, CCR7, and DC-SIGN were determined on intestinal LP DC by flow cytometry as explained in Fig. 1B. (B) The statistical significance of differences between groups was determined using unpaired *t*-test and $p < 0.05$ was regarded as significant ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$). Results are displayed as the mean ± SEM.

clusters within the nucleus. Type-2 DC; are bigger than type 1, with fewer projections, thin chromatin layer surrounding the nucleus, and disaggregated chromatin within the nucleus. Type 3 DC; have long veiled projections and euchromatic nuclei. Therefore, type 1 DC can be considered as immature DC, type 3 DC as mature, and type 2 as an intermediate state.

2.9 Statistical analysis

Two-tailed Fisher's exact test was applied to compare STp presence in the colon from UC and healthy control individuals. Two-way and one-way repeated measures ANOVA, and two-tailed paired tests were applied as stated in the figure legends. In the case of multiple comparisons, subsequent ad hoc Bonferroni correction was applied. $p < 0.05$ was considered significant.

3 Results

3.1 Changes in intestinal DCs in UC

Intestinal DCs were isolated and characterized from healthy controls, and UC patients in remission. Active UC patients were not studied, to assess characteristics constituting UC-specific changes, avoiding nonspecific effects of inflammatory processes on DCs characterization. For phenotype studies (characterization of DCs from UC patients and healthy controls as displayed in Figs. 1–5) the enzymatic digestion protocol was selected for the identification of DCs. For functional studies where DCs were cultured with/without STp (Figs. 6–8), the walkout culture was used for the identification of DCs following conditioning. In all cases human intestinal DCs were identified as HLA-DR$^+$/lineage cocktail$^-$ cells by flow cytometry (Fig. 1A). The majority of these DCs were CD11c$^+$ (myeloid) cells (data not shown). In order to avoid any subjectivity in the interpretation of the results, the percentage and/or intensity ratio (IR) of surface expression on intestinal DCs for each given marker were determined by subtraction of isotype control staining from the corresponding test staining (Fig. 1B and C). Figure 1D shows that LP colonic DCs from inactive UC patients displayed a decreased expression of costimulatory molecules CD80 and CD86 compared with that in healthy control DC.

3.1.1 Reduced expression of langerin on intestinal DCs in UC

Expression of langerin, a molecule involved in antigen capture/uptake (phagocytosis) was determined on intestinal DCs by immunohistochemical analysis of colonic biopsies from healthy controls and UC patients. Langerin$^+$ DCs were located throughout the LP in normal tissues, but tended to be clustered below the epithelium and in deeper regions of the LP in UC (Fig. 2A). All langerin$^+$ cells were counted; there were significantly fewer langerin$^+$ DCs in UC compared with healthy controls (Fig. 2B). Percentage expression of langerin per cell and IR were also determined by flow cytometry (Fig. 2C) and results confirmed the decreased in langerin expression on intestinal DCs from inactive UC patients (Fig. 2D).

3.1.2 Colonic DCs are of immature status in UC patients

Expression of inhibitory receptor IL-T3 and innate pattern recognition receptor DC-SIGN (both characteristic of immature DCs) as well as the lymph node migration marker CCR7 were also analyzed by flow cytometry (Fig. 3A). The proportion of IL-T3$^+$ DCs was significantly increased while CCR7 expression was significantly decreased in inactive UC tissues compared with control tissues (Fig. 3B). Levels of expression

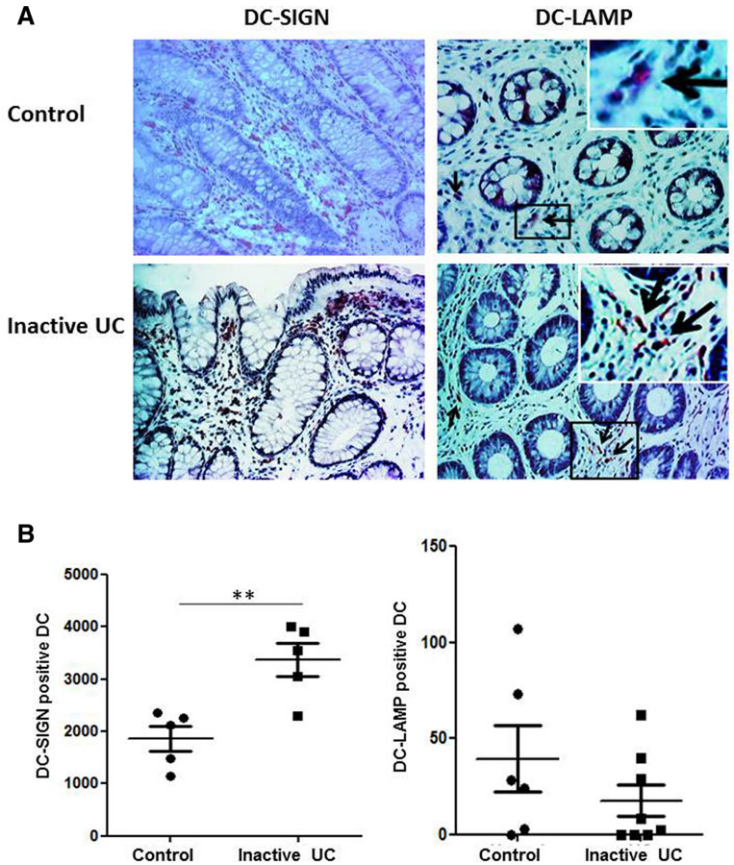

Figure 4. Increased DC-SIGN but not DC-LAMP on LP DC from UC patients. (A) DC-SIGN$^+$ DC were scattered in the LP in healthy controls, whereas in UC was mainly clustered in an area immediately below the epithelium. On the contrary, DC-LAMP$^+$ DCs were very few in number (highlighted with black arrows) and scattered in the LP in both control and UC tissues. A higher magnification of DC-LAMP$^+$ DCs is shown in the inset. (B) Corresponding average numbers of all DC-SIGN$^+$ and DC-LAMP$^+$ DCs per section. Results are displayed as the mean ± SEM. The statistical significance of differences between groups was determined using unpaired *t*-test and $p < 0.05$ was regarded as significant (**$p < 0.01$).

of DC-SIGN per cell, measured by the positive IR, were significantly increased in UC (Fig. 3B). Further immunohistochemical analysis determined that DC-SIGN$^+$ DCs were scattered evenly throughout the LP in healthy controls, but in UC were mainly clustered in an area immediately below the epithelium close to the lumen (Fig. 4A). Numbers of DC-SIGN$^+$ DCs were significantly increased in UC compared with healthy controls (Fig. 4B) in agreement with results from flow cytometry (Fig. 3B). On the contrary, expression of DC-LAMP, a molecule upregulated upon DCs maturation, was minimal (Fig. 4A) and distribution did not change between healthy controls and UC patients (Fig. 4B).

3.1.3 Immature intestinal DC morphology

To further confirm the immature phenotype of intestinal DCs in inactive UC, the maturation status of DCs was also determined by EM, as previously described [25–28]. While there was a mixture of DC at different stages of maturation in all sections examined, no major difference was found in intestinal DC morphology from healthy controls and inactive UC since the majority of DCs in both cases were small cells with short veils relative to their size, small cytoplasmic area, and heterochromatic nuclei with a thick chromatin layer surrounding the nucleus (including chromatic dense clusters) indicating that these cells are type 1 immature myeloid DC [25–28] (Fig. 5) as previously suggested (Figs. 1–4).

3.2 Effects of STp on DCs in UC

In vitro experiments have previously confirmed that STp has the capacity to modulate phenotype and function of human DCs as well as inducing a regulatory effects on gut DCs in healthy controls [22]. Therefore, we determined the capacity of STp to modulate DC functions in UC patients. Intestinal DCs were isolated and characterized from healthy controls and patients with active UC (including both inflamed and noninflamed areas of the colon) to determine any potential immunomodulatory effects of STp specific to either UC or general inflammation.

3.2.1 STp restores expression of costimulatory molecules on DCs in UC

Similar to intestinal DC from UC patients in remission, a significantly lower proportion of DC from noninflamed areas of the UC colon expressed activation molecules and costimulatory molecules compared with that in healthy controls, including CD40 and CD80 (Fig. 6A). Conditioning such intestinal

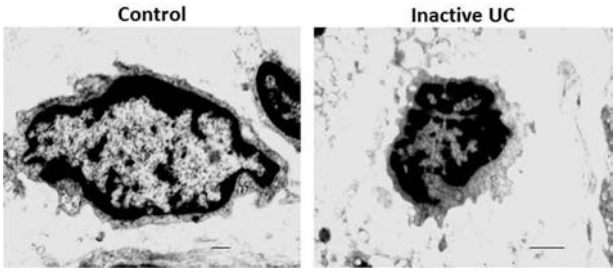

Figure 5. Immature intestinal DC in healthy control and UC tissues. Electron micrographs of DC from the LP showing type-1, myeloid immature DC in normal and UC colonic tissues (Bar = 1μm).

DC from UC patients with STp restored their expression of costimulatory molecules (Fig. 6B).

3.2.2 STp reduces the aberrant expression of TLRs on DCs in UC

TLR2 and TLR4 expression was also studied on intestinal DC (Fig. 7A) to confirm that both markers were aberrantly expressed on intestinal DC in UC compared with healthy controls where their expression was minimal (Fig. 7B). TLR4 was enhanced on DC from both inflamed and noninflamed areas of the gut mucosa in UC, while TLR2 was only enhanced on DC from inflamed areas (Fig. 7B). Our data support previous studies in which it has been proposed that enhanced expression of TLRs on intestinal DC in IBD may contribute to the dysregulated intestinal immune response to bacterial luminal components [11]. Conditioning intestinal DC from UC patients with STp significantly reduced their expression of both TLR2 and TLR4 and partially restored a normal phenotype (Fig. 7C).

3.2.3 STp restores the stimulatory capacity of DCs for allogeneic T-cells in UC

Finally, we examined the capacity of STp to modulate DC ability to generate T-cell proliferative responses during a MLR. Following MLR, dividing T-cells were identified as $CFSE^{lo}$ and T-cell stimulatory capacity of DC was measured as the proportion of dividing T-cells (stimulated with 3% DC of total T-cell numbers in all cases; Fig. 8A). DC from colonic areas in UC (in remission) exhibited a restricted stimulatory capacity for allogeneic T-cells, compared with their healthy control counterparts. However, conditioning these DC with STp restored the stimulatory capacity of DC in UC (Fig. 8B).

4 Discussion

We have analyzed the phenotype and function of human gut DC to demonstrate for the first time, to our knowledge, that in UC DC exhibit an immature phenotype with reduced langerin and CCR7 expression, compared to healthy controls.

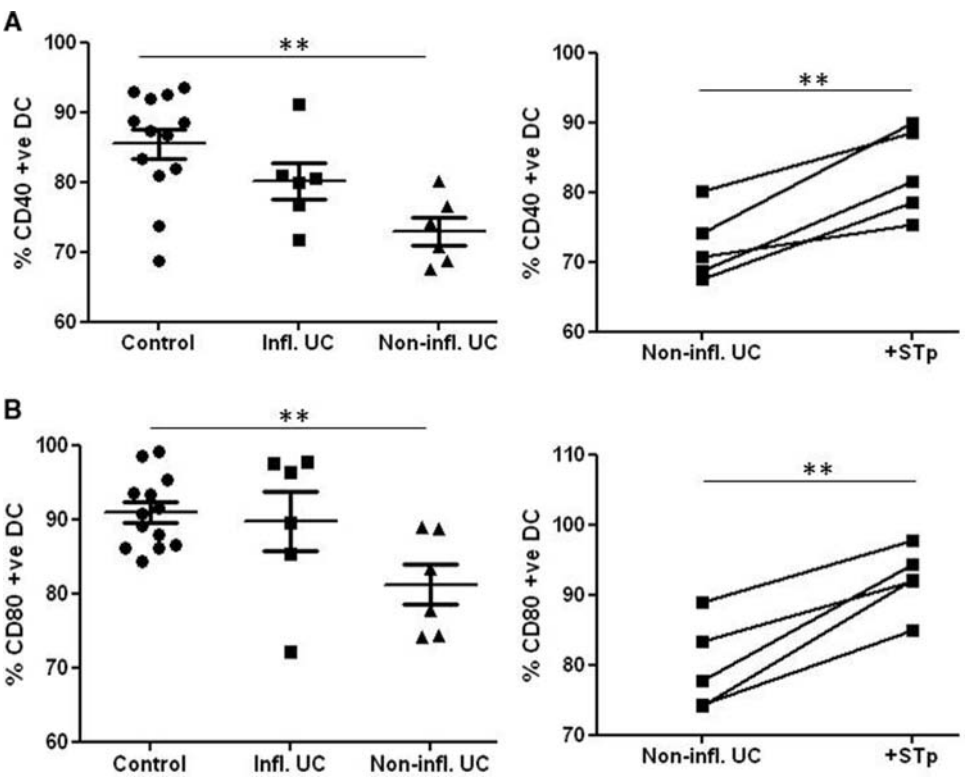

Figure 6. STp effects on the co-stimulatory molecules on intestinal DCs in UC. (A) Expression of activation/maturation markers CD40 and CD80 on intestinal DC from inflamed (Infl) and noninflamed (Non-infl) UC colonic areas from the same patient, compared to colonic tissue from healthy controls. (B) CD40 and CD80 expression on intestinal DC from noninflamed areas treated with STp. Expression for each marker was determined as in Fig. 1B and results displayed as the mean ± SEM. The statistical significance of differences between groups was determined using paired *t*-test and $p < 0.05$ was regarded as significant (**$p < 0.01$).

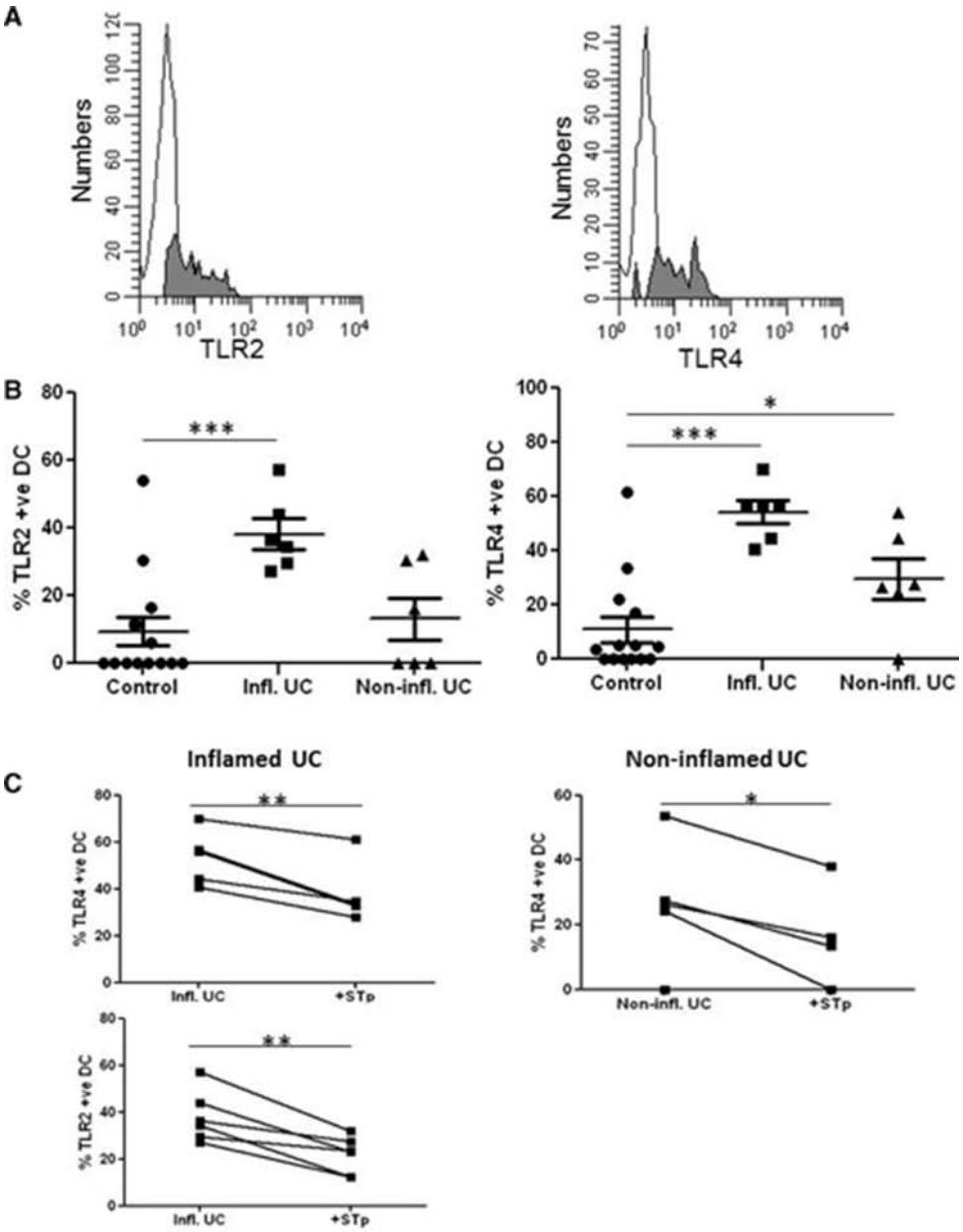

Figure 7. STp reduces the aberrant expression of TLRs on intestinal DCs in UC. (A) Expression of TLR2 and TLR4 on intestinal DC was determined as in Fig. 1B. (B) TLR2 and TLR4 expression was determined on DC from inflamed (Infl) and non-inflamed (Non-infl) colonic tissue from UC patients compared to healthy controls (C) Expression of TLR4 on DC from inflamed and noninflamed colonic tissue and expression of TLR2 on DC from inflamed areas of colonic UC tissue following treatment with STp. Results are displayed as the mean ± SEM. The statistical significance of differences between groups was determined using paired *t*-test and $p < 0.05$ was regarded as significant ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

Moreover, gut DC in UC exhibited a dysregulated function in which they were less able to prime allogeneic T-cell responses compared to control DC. Our data support studies demonstrating immature or partial DC maturation occurs in IBD, and a reduced ability to stimulate primary T-cell proliferation [29, 30]. We have previously demonstrated that STp, a peptide secreted from probiotic strain *L. plantarum*, is present in the colon of healthy controls and has regulatory effects on gut DC in vitro [22]. Here, we show that STp can partially reverse the dysregulated phenotype and function of gut DC found in UC suggesting a role in gut homeostasis for STp that is missing in UC.

Gut DCs have adapted to an environment in which they are continually exposed to a huge antigenic load including commensal microbiota and food antigens. Indeed, intestinal DCs are hyporesponsive to LPS stimulation [4], thus preventing harmful immune responses to commensal flora and providing a potential mechanism of oral tolerance [31, 32]. However, gut DC also exhibit high phagocytic capacity due to continual uptake of antigens from the gut lumen [33]. This sampling of harmless antigens enables tolerogenic T-cell responses to be generated, avoiding inappropriate inflammation. It has been proposed that some autoimmune diseases are due to nonresponsiveness of DC toward antigen cross-reacting with self (auto) antigens, that is, a lack of generated T-cell tolerance. Indeed, DC residing within tissues with antigen cross-reacting with auto-antigens fail to mature [34] and failure of full DC maturation in type-1 diabetes leads to insufficient generation

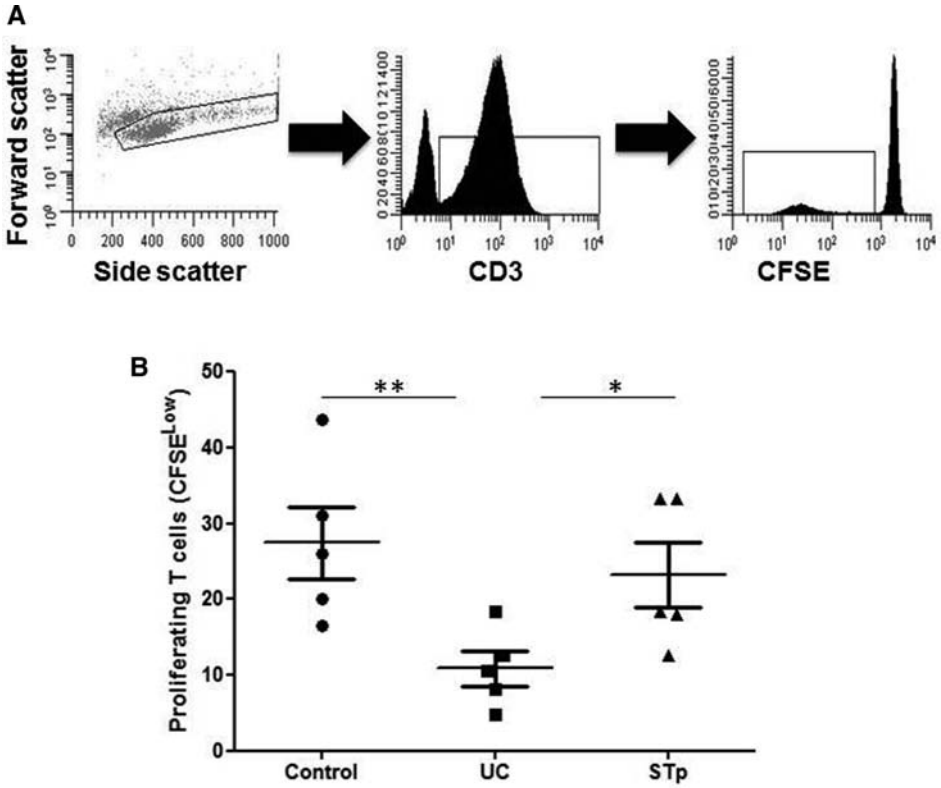

Figure 8. STp restores the stimulatory capacity of DCs for allogeneic T-cells in UC. (A) Identification of dividing T cells in a MLR and (B) effects of STp on the stimulatory capacity of intestinal DC ability to generate T-cell proliferative responses during an MLR.

of regulatory T-cell responses [35]. Our data provide a novel mechanism for UC pathogenesis, in that loss of langerin expression on DC suggestive of loss of phagocytosis, and loss of activation, migration and maturation may lead to reduced ability of DC to generate potentially unresponsiveness in the adaptive immune responses in vivo, contributing to nonspecific inflammation.

UC primarily affects superficial layers of the gut mucosa, unlike Crohn's disease that penetrates the entire bowel wall. Signals controlling DC position within tissues are poorly understood, but likely to involve epithelial-derived chemotactic signals [36]. The redistribution of DC-SIGN$^+$ DC to subepithelial sites near the lumen in UC that we have demonstrated by immunohistochemistry is likely to impact on antigen sampling and modulation of DC by microbial products [37]. Indeed, DC-SIGN binds various microorganisms and initiates innate immunity by modulating TLRs [38, 39]. The altered distribution of immature DC in UC within the gut mucosa in general is likely to be indicative of local involvement of immature DC in disease activity.

The immature phenotype of DC observed in this study may account for the impairment of T-cell activation, resulting in unresponsiveness. It remains to be seen if this immune unresponsiveness by DC would lead to anergic T-cells. These results suggest that the molecular dialog between intestinal bacteria and DC may be mediated by immunomodulatory peptides secreted by commensal bacteria, rather than the bacteria themselves [40], and implicate STp as a potential biomarker of gut homeostasis. It has been proposed recently that the host has no capacity to distinguish between harmful pathogens and commensal bacteria themselves, but that substrates produced by the commensal microbiota actively promote immune tolerance to symbiotic bacteria [41]. In agreement with that STp was found in the colonic microenvironment from 8/10 of the studied healthy controls [22], while when studied in the colon from UC patients in the same conditions it was only found in 3/14 patients with active UC (controls versus UC, Fisher's test, $p = 0.011$; data not shown). Also, there was no difference regarding STp location in the 3 UC patients with detectable levels of STp since that was found on both inflamed (descending colon) and noninflamed areas (transversal/ascending colon) from the same patients. Taken together this suggests that STp is not found as frequently in the colon of UC patients as in that from the healthy controls suggesting a role in gut homeostasis for STp that is missing in UC. However, we do not know if the lack of STp precedes development of UC or on the contrary because of developing UC STp is missing from the colonic microenvironment from these patients.

In summary, our data indicate that as well as promoting immune tolerance [22], STp may partially restore the regulatory phenotype and function of human gut DCs. TLR4 (LPS receptor) expression was increased in UC samples but we confirmed that STp was LPS-free as previously reported [22], so STp effects on gut DCs are likely to be LPS independent. If such effects can be mirrored in vivo, potential implications

are restoration of normal immune function in the gut in UC. Bacteria-derived metabolites, like STp, seem therefore to have a role in gut homeostasis that is missing in UC so they might lead a new era of probiotic products setting the basis for nondrug dietary therapy in IBD.

The author(s) gratefully acknowledge the support of the Biotechnology and Biological Sciences Research Council (BBSRC), this research was funded by the BBSRC Institute Strategic Programme for Gut Health and Food Safety BB/J004529/1. The authors also thank the St Mark's Hospital Foundation, The Association for International Cancer Research (AICR), Scotland, and the Junta de Castilla y León (GRS175/B/07).

The authors have declared no conflict of interest.

5 References

[1] Strober, W., Fuss, I., Mannon, P., The fundamental basis of inflammatory bowel disease. *J. Clin. Invest.* 2002, *117*, 514–521.

[2] Steinman, R. M., Decisions about dendritic cells: past, present, and future. *Annu. Rev. Immunol.* 2012, *30*, 1–22.

[3] Banchereau, J., Steinman, R. M., Dendritic cells and the control of immunity. *Nature* 1998, *392*, 245–252.

[4] Verstege, M. I., ten Kate, F. J., Reinartz, S. M., van Drunen, C. M. et al., Dendritic cell populations in colon and mesenteric lymph nodes of patients with Crohn's disease. *J. Histochem. Cytochem.* 2008, *56*, 233–241.

[5] Rimoldi, M., Chieppa, M., Salucci, V., Avogadri, F. et al., Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells. *Nat. Immunol.* 2005, *6*, 507–514.

[6] Kadowaki, N., Ho, S., Antonenko, S., Malefyt, R. W. et al., Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *J. Exp. Med.* 2001, *194*, 863–869.

[7] Chen, L., Qiu, M., He, W., Huang, A. et al., Functional study of immature dendritic cells co-transfected with IL-10 and TGF-beta 1 genes in vitro. *Mol. Biol. Rep.* 2012, *39*, 6633–6639.

[8] Sallusto, F., Schaerli, P., Loetscher, P., Schaniel, C. et al., Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. *Eur. J. Immunol.* 1998, *28*, 2760–2769.

[9] Knight, S. C., Burke, F., Bedford, P. A., Dendritic cells, antigen distribution and the initiation of primary immune responses to self and non-self antigens. *Semin. Cancer Biol.* 2002, *12*, 301–308.

[10] Chirdo, F. G., Millington, O. R., Beacock-Sharp, H., Mowat, A. M. et al., Immunomodulatory dendritic cells in intestinal lamina propria. *Eur. J. Immunol.* 2005, *35*, 1831–1840.

[11] Hart, A. L., Al-Hassi, H. O., Rigby, R. J., Bell, S. J. et al., Characteristics of intestinal dendritic cells in inflammatory bowel diseases. *Gastroenterology* 2005, *129*, 50–65.

[12] Mann, E. R., Bernardo, D., Al-Hassi, H. O., English, N. R. et al., Human gut-specific homeostatic dendritic cells are generated from blood precursors by the gut microenvironment. *Inflamm. Bowel Dis.* 2012, *18*, 1275–1286.

[13] Bell, S. J., Rigby, R., English, N., Mann, S. D. et al., Migration and maturation of human colonic dendritic cells. *J. Immunol.* 2001, *166*, 4958–4967.

[14] Vuckovic, S., Florin, T. H., Khalil, D., Zhang, M. F. et al., CD40 and CD86 upregulation with divergent CMRF44 expression on blood dendritic cells in inflammatory bowel diseases. *Am. J. Gastroenterol* 2001, *96*, 2946–2956.

[15] Cummings, J. H., Macfarlane, G. T., Macfarlane, S., Intestinal bacteria and ulcerative colitis. *Curr. Issues Intest. Microbiol.* 2003, *4*, 9–20.

[16] Swidsinski, A., Ladhoff, A., Pernthaler, A., Swidsinski, S. et al., Mucosal flora in inflammatory bowel disease. *Gastroenterology* 2002, *122*, 44–54.

[17] Makharia, G. K., Singh, U. B., In the search of a cause of Crohn's disease. *Indian J. Gastroenterol* 2009, *28*, 165–168.

[18] Wang, X., O'Gorman, M. R., Bu, H. F., Koti, V. et al., Probiotic preparation VSL#3 alters the distribution and phenotypes of dendritic cells within the intestinal mucosa in C57BL/10J mice. *J. Nutr.* 2009, *139*, 1595–1602.

[19] Ng, S. C., Plamondon, S., Kamm, M. A., Hart, A. L. et al., Immunosuppressive effects via human intestinal dendritic cells of probiotic bacteria and steroids in the treatment of acute ulcerative colitis. *Inflamm. Bowel Dis.* 2012, *16*, 1286–1298.

[20] Besselink, M. G., van Santvoort, H. C., Buskens, E., Boermeester, M. A. et al., Probiotic prophylaxis in predicted severe acute pancreatitis: a randomised, double-blind, placebo-controlled trial. *Lancet* 2008, *371*, 651–659.

[21] Tsilingiri, K., Barbosa, T., Penna, G., Caprioli, F. et al., Probiotic and postbiotic activity in health and disease: comparison on a novel polarised ex-vivo organ culture model. *Gut* 2012, *61*, 1007–1015.

[22] Bernardo, D., Sanchez, B., Al-Hassi, H. O., Mann, E. R. et al., Microbiota/host crosstalk biomarkers: regulatory response of human intestinal dendritic cells exposed to Lactobacillus extracellular encrypted peptide. *PLoS One* 2012, *7*, e36262.

[23] Coombes, J. L., Maloy, K. J., Control of intestinal homeostasis by regulatory T cells and dendritic cells. *Semin. Immunol.* 2007, *19*, 116–126.

[24] Hsu, S. M., Raine, L., Fanger, H., Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J. Histochem. Cytochem.* 1981, *29*, 577–580.

[25] Patterson, S., Gross, J., Bedford, P., Knight, S. C. et al., Morphology and phenotype of dendritic cells from peripheral blood and their productive and non-productive infection with human immunodeficiency virus type 1. *Immunology* 1991, *72*, 361–367.

[26] Robinson, S. P., Patterson, S., English, N., Davies, D. et al., Human peripheral blood contains two distinct lineages of dendritic cells. *Eur. J. Immunol.* 1999, *29*, 2769–2778.

[27] Bell, S. J., Rigby, R., English, N., Mann, S. D. et al., Migration and maturation of human colonic dendritic cells. *J. Immunol.* 2001, *166*, 4958–4567.

[28] Ng, S. C., Plamondon, S., Al-Hassi, H. O., English, N. et al., A novel population of human CD56 +human leucocyte antigen D-related (HLA-DR+) colonic lamina propria cells is associated with inflammation in ulcerative colitis. *Clin. Exp. Immunol.* 2009, *158*, 205–218.

[29] Al-Hassi, H. O., Bernardo, D., Murugananthan, A. U., Mann, E. R. et al., A mechanistic role for leptin in human dendritic cell migration: differences between ileum and colon in health and Crohn's disease. *Mucosal. Immunol.* 2013, *6*, 751–761.

[30] Bedford, P. A., Todorovic, V., Westcott, E. D., adipose tissue of human omentum is a major source of dendritic cells, which lose MHC Class II and stimulatory function in Crohn's disease. 1. *J. Leukoc Biol.* 2006, *80*, 546–554.

[31] Coombes, J. L., Powrie, F., Dendritic cells in intestinal immune regulation. *Nat. Rev. Immunol.* 2008, *8*, 435–446.

[32] Rescigno, M., Dendritic cells in oral tolerance in the gut. *Cell. Microbiol.* 2011, *13*, 1312–1318.

[33] Rescigno, M., Urbano, M., Valzasina, B., Francolini, M. et al., Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat. Immunol.* 2001, *2*, 361–367.

[34] Knight, S. C., Burke, F., Bedford, P. A., Dendritic cells, antigen distribution and the initiation of primary immune responses to self and non-self antigens. *Semin. Cancer Biol.* 2002, *12*, 301–308.

[35] Jansen, A., van Hagen, M., Drexhage, H. A., Defective maturation and function of antigen-presenting cells in type 1 diabetes. *Lancet* 1995, *345*, 491–492.

[36] Yang, D., Chertov, O., Bykovskaia, S. N., Chen, Q. et al., Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. *Science* 1999, *286*, 525–528.

[37] Konstantinov, S. R., Smidt, H., de Vos, W. M., Bruijns, S. C. et al., S layer protein A of *Lactobacillus acidophilus* NCFM regulates immature dendritic cell and T cell functions. *Proc. Natl. Acad. Sci. USA* 2008, *105*, 19474–19479.

[38] den Dunnen, J., Gringhuis, S. I., Geijtenbeek, T. B., Innate signaling by the C-type lectin DC-SIGN dictates immune responses. *Cancer Immunol. Immunother.* 2009, *58*, 1149–1157.

[39] Geijtenbeek, T. B., Van Vliet, S. J., Koppel, E. A., Sanchez-Hernandez, M. et al., Mycobacteria target DC-SIGN to suppress dendritic cell function. *J. Exp. Med.* 2003, *197*, 7–17.

[40] Hoarau, C., Martin, L., Faugaret, D., Baron, C. et al., Supernatant from bifidobacterium differentially modulates transduction signaling pathways for biological functions of human dendritic cells. *PLoS One* 2008, *3*, e2753.

[41] Round, J. L., Lee, S. M., Li, J., Tran, G. et al., The Toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. *Science* 2011, *332*, 974–977.

