TITLE: IL-6 promotes immune responses in human ulcerative colitis and induces a skin homing phenotype in dendritic cells and T-cells they stimulate.

AUTHORS: David Bernardo[1], Sara Vallejo-Díez[2], Elizabeth R. Mann[1], Hafid O. Al-Hassi[1], Beatriz Martínez-Abad[2], Enrique Montalvillo[2], Cheng T. Tee[1], Aravinth U. Murugananthan[1], Henar Núñez[3], Simon T. C. Peake[1], Ailsa L. Hart[4], Luis Fernández-Salazar[5], Jose A. Garrote[6], Eduardo Arranz[2], Stella C. Knight[1].

AFFILIATIONS:
1. Antigen Presentation Research Group. Imperial College London, Northwick Park & St Mark´s Campus. Watford Road. Harrow. United Kingdom
2. Mucosal Immunology Lab. Department of Paediatrics & Immunology. Universidad de Valladolid, IBGM-CSIC. Spain.
3. Service of Gastroenterology. Hospital Universitario Rio Hortega. Valladolid. Spain.
4. Department of Gastroenterology. St Mark´s Hospital. North West London Hospitals NHS Trust. Harrow. United Kingdom
5. Gastroenterology service. Hospital Clínico Universitario de Valladolid. Valladolid. Spain.
6. Genetics and Molecular Biology Lab. Service of Clinical Laboratory. Hospital Universitario Río Hortega. Valladolid. Spain.

KEYWORDS:
dendritic cells, ulcerative colitis, human, IL-6, extra-intestinal manifestations.

CORRESPONDING AUTHOR
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.
Phone +44 (0) 20 8869 3532; Fax +44 (0) 20 8869 3532
Email s.knight@imperial.ac.uk

ABBREVIATIONS
DC: Dendritic cells
IBD: Inflammatory bowel disease
iNKT: Invariant Vα24 restricted T-cells
PBMC: Peripheral blood mononuclear cells
RA: Retinoic acid
SN: Culture supernatants
UC: Ulcerative colitis

RUNNING TITTLE: IL-6 mediates dysregulated DC conditioning in UC

SUMMARY

Dendritic cells (DC) control the type and place of immune responses. Ulcerative colitis (UC) is considered a TH2 disease mediated by IL-13 where up to 1/3 of the patients can develop extra-intestinal manifestations. Colonic biopsies from inflamed and non-inflamed areas of UC patients were cultured in vitro and their supernatants were used to condition human blood enriched DC from healthy controls. Levels of IL-13 in the culture supernatants were below the detection limit in most cases and the cytokine profile suggested a mixed profile rather than a TH2 cytokine profile. IL-6 was the predominant cytokine found in inflamed areas from UC patients and its concentration correlated with the Mayo endoscopic score for severity of disease. DC conditioned with non-inflamed areas from UC patients acquired a regulatory phenotype with decreased stimulatory capacity. However, DC conditioned with inflamed areas acquired a pro-inflammatory phenotype, increased expression of skin homing CCR8, did not decrease their stimulatory capacity for T-cells and primed them with the skin-homing CLA molecule in an IL-6 dependent mechanism. Our results highlight the role of IL-6 in UC and question the concept of UC as a TH2 disease and the relevance of IL-13 in its aetiology.

INTRODUCTION

The gastrointestinal tract is in contact with a wide variety of commensal microbiota and diverse pathogens, and therefore requires a balance to be maintained between immunity and immune tolerance; the lack of immune responses, or immune tolerance, against food antigens and/or the commensal microbiota is essential to keep the homeostasis of the gastrointestinal tract [1].

Ulcerative colitis (UC) is a form of inflammatory bowel disease (IBD), traditionally related to a TH2 cytokine profile mediated by IL-13 [2, 3], where immune homeostasis of the gastro-intestinal tract is compromised. Up to 1/3 of UC patients can develop extra-intestinal manifestations being the skin one of such tissues [4, 5].

Dendritic cells (DC), "*commanders-in-chief*" of the immune system, are the most potent antigen presenting cells. DC determine the nature and type of immune responses [6 7]. Intestinal DC control immune tolerance in the gastrointestinal tract [8-10]. DC also maintain immune responses localized to specific tissues, where they imprint specific tissue-homing profiles on stimulated T-cells [11]. Retinoic acid (RA) (the active form of vitamin A following dehydrogenization by the RALDH2 enzyme) controls some of the mechanisms of immune homeostasis of the gut [12-14]. RA producing DC mediate the IgA switching of B-cells [15], the generation T-cells with regulatory phenotype [10] and the imprinting of gut-homing markers on B- and T-cells [16,17], thereby keeping tolerogenic immune responses compartimentalized to the gastrointestinal tract. A tolerogenic role has also been recently described for fracktalkine (CX3CR1) since knockout mice failed to develop oral tolerance [18]. Invariant Vα24 restricted T-cells (iNKT cells) also play a role in oral tolerance although their exact role remains unclear [19-21].

In healthy individuals, gut microenvironment controls the phenotype and function of human DC. Thus, tolerogenic "gut-like" DC can be generated when they are exposed to such microenvironment [22-26]. In UC patients immune homeostasis in the gastro-intestinal tract is compromised. Compared with biopsies from healthy controls, biopsies from inflamed and non-inflamed areas of the colon from UC patients exhibit increased production of pro-inflammatory cytokines, with higher levels within inflamed areas. However the expression of mediators of tissue damage was restricted to inflamed areas [27].

We hypothesised that local factors controlling intestinal homeostasis in UC patients are either lost or masked by ongoing inflammation in inflamed areas of the gut, driving DC towards a pro-inflammatory phenotype, and orchestrating the dysregulated immune response in the gut. We characterized the local expression of soluble cytokines and the gene expression profile of molecules involved in intestinal homeostasis in the gut. Secondly, we studied the effect of conditioning human blood enriched DC with the intestinal microenvironment from both inflamed and non-inflamed areas of the gut, from UC patients. Our results confirmed that non-inflamed areas of the gut from UC patients rendered DC less stimulatory. However, DC conditioned with inflamed areas from the same patients acquired an increased production of pro-inflammatory cytokines, a skin-homing profile and imprinted a skin-homing phenotype on T-cells in an IL-6 dependent mechanism.

RESULTS

Increased pro-inflammatory cytokine profile in inflamed areas of UC patients.

Cells present in biopsies from inflamed areas of UC patients compared with paired healthy non-inflamed areas produced higher levels of pro-inflammatory cytokines (Figure 1A). Neither gender, nor age nor extension of the disease in the patients showed a significant effect on the cytokine production (data not shown). IL-13, considered to be the effector cytokine in UC [2,3], was not differentially secreted between inflamed and non-inflamed areas of the gut of UC patients and in most of the cases (10 out of 11 from healthy and 6 out of 11 from inflamed areas) it was below detection limit. IL-13 secretion was also below detection limit in inflamed areas from 2 of the 3 patients with a Mayo endoscopic score of 3 and therefore it did not correlate either with the severity of the disease. IL-6 was the predominant cytokine found in inflamed areas of UC patients (2760±813 pg/ml) (Figure 1B) and its concentration correlated with the Mayo endoscopic score (Figure 1C) being the only cytokine displaying such characteristic. TH2-related cytokines, like IL-4 and IL-7, were also increased in inflamed areas. However their low concentration (IL-4: 3.8±0.7 pg/ml; IL-7: 21.3±3.3 pg/ml) compared with IL-6 (2760±813 pg/ml) and other TH1 related cytokines that were also increased in inflamed areas like IFNα, (245.9±27.3 pg/ml), IFNγ (328.5±72.63 pg/ml) and TNFα (153.1±36.5 pg/ml) suggest a mixture profile rather than a TH2 cytokine profile (Figure 1B).

IL-6 controls the dysregulated cytokine profile in UC patients.

Since IL-13 is considered to be the effector cytokine in UC [2,3] we performed blocking experiments in our culture system. Addition of anti IL-13 to inflamed areas of UC patients did not decrease the secretion of any of the assayed cytokines and on the contrary it increased further the secretion of IL-4 and IL-17 (Figure 2). These results might suggest that in UC patients the role of IL-13 could be elicited through an auto and/or paracrine manner which may explain why such cytokine was not found in the

culture supernatants (Figure 1A) and therefore why its effect was not abrogated by soluble blocking antibody (Figure 2). Addition of soluble recombinant IL-13 to healthy intestinal areas of UC patients failed to increase the secretion of IFNγ, TNFα, IL-4 and IL-6 as seen on inflamed areas and only restored the secretion of IFNα, IL-7 and IL-17 (Figure 3). Interestingly, IL-17 was induced both in healthy areas exposed to IL-13 and in inflamed areas blocked with anti IL-13 probably reflecting different mechanisms of control.

Opposed to IL-13, IL-6 was the predominant cytokine found in the culture supernatants of inflamed areas in UC patients (Figure 1B) and its concentration correlated with the Mayo endoscopic score (Figure 1C). We explored therefore its role controlling the intestinal cytokine milieu in UC patients. Anti IL-6 supplementation to biopsy cultures from inflamed areas of UC patients effectively blocked the available IL-6 in the biopsy culture supernatants (Figure 4). Secreted cytokines which were up-regulated in inflamed areas from UC patients (Figure 1A) were decreased following biopsy incubation in the presence of anti IL-6 (Figure 4) and their levels restored (IFNα, TNFα, IL-7) or even decreased (IL-4, $p<0.01$; and IFNγ, $p<0.05$) compared with those found in healthy areas. Similarly, we cultured non-inflamed intestinal areas from UC patients in the presence of IL-6. After IL-6 stimulation, all assayed secreted cytokines (except regulatory IL-10), were induced in the biopsy cultures (Figure 5) to the levels displayed in the paired inflamed areas (Figure 1A) while IFNγ and IL-4 acquired higher values than those identified in inflamed areas from such patients ($p<0.001$ in both cases). Together, these findings highlight the central role of soluble IL-6 controlling the dysregulated cytokine milieu found in inflamed areas of UC patients in the absence of any external challenge and diminish the role of soluble IL-13 in our culture system.

Inflamed areas of the gut from UC patients have decreased expression of Cx3CR1 and RALDH2.

We then studied the expression of several mRNA molecules related to mechanisms of intestinal tolerance, such as bacterial load (16s), Muc2 (main protein from the mucus layer), FoxP3 (representative of T-cells with a regulatory phenotype), Vα24 (invariant chain of Vα24-restricted invariant NKT cells), Cx3CR1 (fractalkine) and RALDH2 (necessary to metabolize retinoic acid from dietary vitamin A) (Figure 6). Neither gender, nor age nor extension of the disease had any effect in the mRNA expression profile of any of the assayed molecules (data not shown). None of the assayed molecules correlated with the Mayo endoscopic score for severity of the disease or with the concentration of secreted cytokines (data not shown). Muc2, 16s, FoxP3 and Vα24 expression were not differentially expressed between sampling areas. However, inflamed intestinal areas from UC patients had decreased mRNA expression of both RALDH2 and CX3CR1. Having characterized both the local cytokine milieu (Figure 1) and the gene expression profile (Figure 6) of both inflamed and non-inflamed areas from UC patients, we next studied the effect of such intestinal microenvironment in conditioning the phenotype and function of human blood enriched DC.

DC conditioned with inflamed areas of the gut from UC patients become skin-homing not-regulatory DC.

DC from each healthy control were conditioned in the presence of basal medium and SN from paired healthy and inflamed colonic areas from a single UC patient after confirming that this is a valid approach to study the effect of the tissue microenvironment on the phenotype and function of DC [26]. Following DC conditioning with intestinal microenvironment from inflamed and healthy intestinal areas of UC patients, there was no differential cell survival (data not shown). DC conditioned with healthy areas from UC patients acquired a regulatory phenotype while those conditioned with inflamed areas from the same patients did not. Thus, although exposure to both inflamed and healthy areas increased CD40 expression on DC together with an increased potential to migrate to the lymph nodes (CCR7 up-

regulation) (Figures 7A and 7B), HLA-DR expression was specifically decreased in DC exposed to non-inflamed areas from the gastrointestinal tract (Figure 7A and 7B). Expression of other activation/maturation markers (CD83, CD86) was unaffected, as were TLR2 and TLR4 (data not shown). Since CX3CR1 mRNA expression was specifically decreased in inflamed areas, we studied expression on DC following conditioning with either inflamed or healthy intestinal microenvironments. CX3CR1 expression on DC was increased on DC exposed to both microenvironments with no statistically significant differences (Figure 7A and 7B).

We have previously identified that when cultured "in vitro" in culture medium only, DC lose expression of their homing markers [26]. However, if DC are cultured in the presence of a tissue microenvironment, DC acquire characteristics of local tissue DC, including the expression of specific tissue-associated homing markers. Thus, a gut-homing profile can be induced on DC when exposed to gut SN from healthy colonic biopsies [26]. However DC exposed to gut SN from UC patients (either inflamed or healthy areas) did not acquire a gut-homing profile. Thus gut-homing β7 and gut-retaining CD103 (mean expression below 10% in all cases) were not induced in any case. Similarly, the expression of skin-homing markers CLA and CCR4 (mean expression below 10% in all cases) did not change either (data not shown). However, the skin-homing chemokine receptor CCR8 was specifically induced on DC following conditioning with inflamed areas from UC patients (Figures 7A and 7B), providing DC with potential to migrate to cutaneous sites.

Non-inflamed areas from UC patients promote a regulatory cytokine phenotype on DC.

Since DC have been cultured with cultured SN that are loaded with cytokines (Figure 1A), assessing cytokine secretion by DC is not a feasible approach to study their cytokine production. Therefore, we studied their natural ongoing intracellular cytokine

production following conditioning with intestinal microenvironments. This protocol has been validated by our group [8] and allows us to assess the natural on-going cytokine production (without external PMA and/or ionomycine stimulus) of DC in the absence of any external challenge.

None of the assayed cytokines in the culture SN correlated with the ongoing cytokine production of DC following SN-conditioning (data not shown). When exposed to a non-inflamed microenvironment, DC acquired a regulatory cytokine profile. This was demonstrated by the decreased ongoing production of pro-inflammatory cytokines IL-12 and IL-6, and increased regulatory cytokine IL-10, when compared with DC exposed to paired inflamed areas (Figures 8A and 8B). Ongoing production of TGFβ was not changed in any case. Therefore, while DC conditioned with healthy SN acquired a regulatory cytokine profile, those DC conditioned with inflamed SN from the same donors were driven towards a TH1/TH17 cytokine profile.

DC conditioned with inflamed microenvironment increase their stimulatory capacity and prime T-cells with a skin-homing profile.

The stimulatory capacity of DC was specifically modified following SN conditioning (Figures 9A and 9B). When total numbers of DC were increased in the mixed leukocyte reaction higher T-cell proliferative responses were obtained (Figure 9B). β7 integrin is induced in all stimulated human T-cells irrespectively of the source of human DC while CLA expression on stimulated T-cells is dependent on the tissue source of human DC [26]. DC conditioned with healthy intestinal areas from UC patients decreased their stimulatory capacity and promoted the generation of gut-homing T-cells (Figure 9C) since stimulated (CFSE^{low}) T-cells maintained gut-homing β7 integrin and decreased skin-homing CLA expression compared with un-conditioned (basal) DC. Similar results have been previously reported when using culture SN from healthy controls [26]. However, when DC had been conditioned with inflamed areas from the same patients,

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

DC did not decrease their stimulatory capacity (Figure 9B). Also, their acquired skin-homing capacity (Figure 7B) was reflected in an increased capacity to prime skin-homing CLA on stimulated T-cells compared to DC conditioned with healthy areas from the same patients (Figure 9D).

IL-6 plays a central role in the dysregulated immune response in UC patients.

IL-6 was the predominant cytokine found in inflamed areas from UC patients (Figure 1B) and its concentration correlated with the Mayo endoscopic score for severity of disease in such areas (Figure 1C). Also, natural ongoing production of IL-6 in DC correlated with the stimulatory capacity of DC for T-cells (Figure 10A) being the only studied cytokine which displayed such characteristics. Therefore we studied the effect of IL-6 blocked inflamed supernatants from UC patients, were the cytokine profile was restored to normal (Figure 4), on DC phenotype and function. DC conditioned with such IL6-blocked microenvironment decreased their stimulatory capacity (Figure 10B); generation of stimulated skin-homing T-cells was also inhibited (Figure 10D). To confirm that IL-6 controls immune inflammation in UC patients we challenged non-inflamed areas from such patients with IL-6. Following IL-6 supplementation a pro-inflammatory cytokine microenvironment was induced (Figure 5) and protective MUC2 mRNA expression was decreased while Vα24 mRNA expression was increased which suggests specific iNKT cell proliferation (data not shown). When blood DC were exposed to such an IL-6 rich microenvironment, they decreased their ongoing production of IL-10 and failed to decrease their stimulatory capacity (Figure 10C). They also acquired the capacity to prime stimulated T-cells with an increased skin homing capacity (Figure 10D). Together, our results indicate a central role of human intestinal IL-6 in orchestrating immune responses by controlling the pro-inflammatory milieu and the subsequent phenotype and function of DC.

DISCUSSION

We have identified that in humans inflamed areas of the gut from UC patients have increased production of soluble pro-inflammatory cytokines -where IL-6 was the predominant cytokine found in inflamed areas from UC patients- and have decreased mRNA expression of RALDH2 and CX3CR1. Such an intestinal microenvironment conditions the phenotype and function of blood enriched DC. Non-inflamed areas from UC patients biased DC towards a regulatory phenotype with decreased stimulatory capacity. Paired DC conditioned with inflamed areas from the same patients increased expression of skin homing CCR8, did not decrease their stimulatory capacity and primed T-cells with the skin-homing CLA molecule in an IL-6 dependent mechanism. Our results question whether UC really is a TH2 based disease and if mirrored in vivo, they provide a molecular explanation to the generation of skin extra-intestinal manifestations in UC patients.

DC are sentinels and sensors of the immune system and their phenotype and function are dependent on the tissue microenvironment [28]. The tissue microenvironment confers on DC the capacity to react quickly to the presence of an innate stimulus, also activating the mechanisms of a secondary antigen-specific adaptive immune response following antigen presentation. If DC are cultured in conditioned-medium which had a previous culture of a human tissue and/or a human cell line, DC acquire a tissue-like phenotype and express characteristics of resident DC in such a tissue. Thus, human gut-like DC can be generated from blood precursors following such a protocol [22-26]. Rather than studying the phenotype and function of human tissue DC when the amount of tissue is scarce, and therefore functional experiments are not feasible, such methodology can be employed to study the effect of tissue microenvironment on functions of more readily available blood DC. Following conditioning with tissue microenvironment from healthy or inflamed colonic areas from UC patients, DC were matured in both cases as assessed by CD40 and CRR7 upregulation (Figure 7).

However, and similar to DC conditioned with a colonic microenvironment from healthy individuals, DC conditioned with non-inflamed areas from UC patients acquired a regulatory or less stimulatory phenotype and T-cell that they stimulated acquired a gut-homing profile characterized by a decreased CLA expression [26]. On the contrary DC conditioned with inflamed areas from UC patients were matured towards a non-regulatory phenotype since they decreased ongoing production of regulatory IL-10 and increased production of pro-inflammatory IL-12 and IL-6 compared to DC conditioned with healthy areas from the same patients. Similar observations regarding the capacity of intestinal IL-6 to promote immune responses in Crohn's disease patients have been recently described [29]. Future studies should identify the specific mechanisms and signalling pathways through which IL-6 mediated effects are established on DC, and whether IL-6 on its own (in the absence of an intestinal microenvironment) has the capacity to prime DC and/or stimulated T-cells with a skin-homing profile.

Given the dual capacity of DC to control both immunogenic and tolerogenic immune responses recent mouse studies have suggested that two different tolerogenic (CD103+) and pro-inflammatory (CX3CR1+) non-overlapping DC sub-populations co-exist in the gut [30-32]. These findings suggest that DC responsible for regulatory and inflammatory responses might be of distinct origin, phenotype and function at resting and inflammatory conditions. If some circulating contaminating monocytes have remained in our blood enriched DC and/or if the same functional dichotomy applies in human circulating DC it might be possible that different DC subtypes are involved in mediating "regulatory" and "inflammatory" responses in our culture system when exposed to different colonic microenvironments. However such studies obtained from mouse ileum remain to be validated in the human context and preliminary experiments suggest that such separation of populations may not be entirely true in the human colon since CX3CR1+ DC are virtually absent from the colonic lamina propria (data not shown). On the contrary it seems more feasible that DC phenotype and function are

conditioned by the surrounding microenvironment to perform a tolerogenic or non-tolerogenic function depending on the required needs. In agreement with our findings, intestinal "tolerogenic" antigen presenting cells are reverted towards a pro-inflammatory phenotype when exposed to an inflamed microenvironment in mice models of colitis [33,34]. Therefore, it seems likely that the UC inflamed microenvironment will modulate "in vivo" the phenotype of newly arrived DC towards a pro-inflammatory phenotype.

Although healthy intestinal areas from UC patients have decreased expression of pro-inflammatory mediators compared with expression in paired inflamed areas (Figure 1A), such mediators are still higher than those from biopsies of healthy controls [27]. That may explain why DC conditioned with such non-inflamed areas from UC patients failed to increase expression of gut-homing markers on DC such as we have described when DC are exposed to intestinal culture supernatants form healthy controls [26]. In the present manuscript we have also shown that inflamed areas from UC patients had decreased mRNA expression of both CX3CR1 and RALDH2 (Figure 6) compared with expression in inflamed areas. Although through our approach we do not know in which cell type they are expressed, both molecules are essential in mechanisms of intestinal homeostasis and oral tolerance [12-14,18]. Whether such decreased expression is a consequence of the pro-inflammatory cytokine microenvironment (Figure 1A) or on the contrary such pro-inflammatory phenotype is a consequence of decreased CX3CR1 and/or RALDH2 (among other possible factors) is an issue that has to be answered. Neither CX3CR1 nor RALDH2 recovered their normal expression levels in inflamed areas in the presence of blocking IL-6. Similarly, they were not decreased in healthy areas exposed to IL-6 (data not shown) in spite of the induced pro-inflammatory microenvironment (Figure 5). Together, these results suggest that the decreased local expression of CX3CR1 and RALDH2 in UC patients may be a pre-disposing factor,

rather than a consequence, for local inflammation and tissue damage. However such hypothesis should be explored further since some authors have recently described a pro-inflammatory role for retinoic acid at low concentrations in the presence of IL-15 [35]. Also, the role of local iNKT cannot be discarded. Such cell population can display both regulatory and pro-inflammatory properties [36] and they have been proposed as the original source of pro-inflammatory cytokines in UC [3]. Following IL-6 challenge of healthy tissue from UC patients we have identified increased Vα24 expression which suggests an iNKT cell expansion. Future studies should define any role for tissue iNKT in such pathology.

The causes of extra-intestinal manifestations in IBD are poorly understood and have usually been considered as a consequence of a miss-matched immune response of the target tissue. However, such extra-intestinal manifestations may be a consequence of a dysregulation in the lymphocyte homing pathways [37-39]. In agreement, our findings demonstrate the effect of the tissue microenvironment in controlling DC phenotype and function, and provide an explanation for the development of extra-intestinal manifestations in UC patients due to a dysregulated homing response. Thus, following conditioning with inflamed areas from UC patients DC acquired a non-regulatory skin-homing phenotype together with an increased capacity to generate skin-homing T-cells through an IL-6 dependent mechanism. Supporting our data, aberrant expression of skin homing markers on peripheral blood DC from IBD patients suffering from skin manifestations has been recently demonstrated. Also, DC from active UC patients increase CLA expression on T-cells that they stimulate (Mann ER, personal communications). Future experiments should identify the different nature (if any), phenotypic and/or functional particularities of such DC which are likely to be involved in the dysregulation of leukocyte trafficking and the development of extra-intestinal manifestations in IBD.

UC has been traditionally considered as a TH2 disease, with IL-13 as the effector cytokine [2-3]. Although IL13 mediates damage of intestinal epithelial cells in murine models of colitis [40] there is no formal proof that IL-13 is a pathological relevant cytokine in UC in humans context since mouse models do not always reflect the exact mechanisms and/or cytokine profile found in the human context [3, 41]. Others have described a role of IL-13 in human UC pathogenesis characterized as mRNA expression in human colonic biopsies [42] or as IL-13 secretion of total lamina propria mononuclear cells following an "in vitro" stimulation [2]. Opposed to such studies, our assessment of the local cytokine milieu on human samples has been performed in the absence of any external stimulus which just measures the spontaneous cytokine production and does not support this view in our set of patients. Moreover, inflamed and not-inflamed intestinal biopsies from UC patients did not differ regarding production of soluble IL-13 and in most cases its concentration was below the limit of detection (Figure 1A). Blocking IL-13 in the culture system did not have a major effect on the concentration of cytokines in inflamed areas from UC-patients (Figure 2) while addition of recombinant IL-13 to healthy intestinal areas (Figure 3) did not have a comparable effect in production of pro-inflammatory cytokines to that achieved by IL-6 stimulation (Figure 5). IL-6 was the predominant cytokine found in inflamed areas from UC patients and the subsequent most predominant cytokines were related with a TH1 profile (Figure 1B). We are aware that the functional potency of secreted cytokines is highly dependent on the affinity and density of the specific cytokine receptors and therefore a given cytokine might be "ignored" with lower specific receptor densities [43]. However, IL-6 also correlated with the Mayo endoscopic score for severity of the disease (Figure 1C) while its blockage in inflamed areas reverted the inflammation and its supplementation to healthy areas mimicked the inflammation. Together, our data questions the relevance of IL-13 and highlights the role of IL-6 in UC such as we and others have described [27,44,45] suggesting that UC may be characterized by a mixture profile rather than a TH2 cytokine profile [46,47]. Therefore, although current

therapies for UC patients involve systemic administration of anti TNFα, anti IL-6 (alone

and/or in combination with TNFα) is revealed as a potentially more efficient approach

as some authors have recently reported [48-50].

In summary, our data questions the concept of UC as a TH2 disease, questioning the

relevance of IL-13 in its etiology and highlighting the role of IL-6 as a central cytokine

controlling the local immune response. Our results confirmed that the tissue

microenvironment conditions the phenotype and function of DC. Thus, DC conditioned

with non-inflamed areas from such patients acquired a tolerogenic "gut-like" phenotype

with decreased stimulatory capacity. However, DC conditioned with inflamed areas

from the same patients did not decrease their stimulatory capacity and increased their

skin-homing phenotype and skin-homing imprinting capacity on T-cells in an IL-6

dependent mechanism. If mirrored in vivo, this increased potential for skin homing

provides an explanation for the generation of extra-intestinal manifestations in the skin

in UC patients and provides us with new targets for immunomodulation.

MATERIAL AND METHODS

Colonic samples and biopsy culture

Colonic biopsies were obtained at colonoscopy from active UC patients following informed consent after ethical approval from the Hospital Clínico Universitario de Valladolid (Spain) and the Outer West London Research Ethics Committee (United Kingdom). A total of 12 UC patients were sampled. Only one patient had skin extra-intestinal manifestation of the disease (erythema nodosum) at the time of sample taking. Total extension of the lesion and the Mayo endoscopic score (from 0 to 3) for severity of the disease were determined for each patient (Table 1). Paired samples from the same patient were collected from macroscopically inflamed and macroscopically healthy non-inflamed areas of the gut in ice-chilled PBS. Biopsy culture was started within an hour. Biopsies were cultured in 1.5ml of complete medium (Dutch modified RPMI 1640 (Sigma-Aldrich, Dorset, UK) containing 100u/mL penicillin/streptomycin, 2mM L-glutamine, 50µg/mL gentamicine (Sigma-Aldrich) and 10% foetal calf serum (TCS cellworks, Buckingham, UK) for 24 hours in 12 well culture dishes (1 biopsy/well) (37ºC, 5% CO_2, high humidity). In some cases, extra biopsies from inflamed areas were cultured in the presence of anti IL-6 (0.5µg/ml, R&D) or anti IL-13 blocking antibody (2.5µg/ml, R&D) while in others, biopsies from healthy areas were cultured in the presence of recombinant IL-6 (50ng/ml, R&D) or IL-13 (50ng/ml, R&D). All biopsies had similar size and weight and were randomly cultured in the different culture conditions in order to standardize the error in the supernatants´ cytokine content which might be derived from different biopsy size. After 24 hours, media from biopsy culture were centrifuged and cell-free supernatants (SN) collected, while tissue was embedded in RNAlater (Ambion) and snap-frozen. Total RNA was isolated from each biopsy using the TRIZOL® reagent according to the protocol provided by the manufacturer. Reverse transcription was carried out by using the SuperScript® First-Strand Synthesis System for reverse Transcriptase (RT)-PCR Kit (Invitrogen, Life Technologies, USA) using random hexamers as primers.

Blood samples and biopsy conditioning

Human peripheral blood was collected from healthy volunteers with no known autoimmune or inflammatory diseases, allergies or malignancies, following informed consent. Peripheral blood mononuclear cells (PBMC) were obtained by centrifugation over Ficoll-Paque Plus (Amersham Biosciences, Chalfont St. Giles, UK). Human blood enriched DC were obtained following NycoPrep™ centrifugation of overnight cultured PBMC. This protocol has been characterised in detail in previous studies from our laboratory as a way to obtain fresh human blood enriched DC [51,52]. Obtained cells display morphological characteristics of DC (both at optical microscopy and electron microscopy), express HLA-DR and are potent stimulators of naïve T-cells.

DC from each donor were incubated for 24 hours (0.5 million cells/ml) in the presence of basal medium and SN from paired healthy and inflamed colonic areas from a single UC patient. Such approach provides a tool to study the effect of the intestinal microenvironment in the conditioning of the phenotype and function of human DC [26].

Quantitative Polymerase Chain Reaction.

mRNA levels of 16s, Muc2, FoxP3, Vα24, Cx3CR1, RALDH2 and GADPH (*housekeeping* gene), were measured by real-time PCR by using a LightCycler® instrument (Roche Applied Science, Mannheim, Germany). Reactions were performed using the FastStart SYBR Green MasterMix (Roche) with thermolabile Uracil DNA Glycosylase (UDG) (Roche) to prevent carry-over contamination. Primer sets and PCR conditions are described in table 2. mRNA levels are expressed as the ratio molecule/ GADPH in arbitrary units (U).

Cytokines in culture supernatants.

Cell-free culture supernatants were analyzed by using a multiplex assay (Biorad, Hercules, CA) on a Luminex TM platform (Austin, TX), following the manufacturer´s instructions, for the concentration of interferon α (IFNα) [detection limit (D.L.) 125 pg/ml], IFN gamma (IFNγ [D.L. 4.04 pg/ml], tumour necrosis factor α (TNF-α) [D.L. 3.69 pg/ml], interleukin (IL)-4 [D.L. 0.43 pg/ml], IL-6 [D.L. 25.7 pg/ml], IL-7 [D.L. 3.18 pg/ml], IL-10 [D.L. 1.39 pg/ml], IL-13 [D.L. 3.74 pg/ml] and IL-17 [D.L. 12.63 pg/ml]. Those values below the level of detection were reported as being equal to that.

Antibody labelling

Table 3 shows the specificity, clone and fluorochrome of the monoclonal antibodies used. Cells were labelled in PBS containing 1mM EDTA and 0.02% sodium azide (FACS buffer). Labelling was performed on ice and in the dark for 20'. Cells were washed twice in FACS buffer, fixed with 1% paraformaldehyde in 0.85% saline and stored at 4^0C prior to acquisition on the flow cytometer (within 48 hours). Appropriate isotype-matched control antibodies were purchased from the same manufacturers. For intracellular staining, cells were fixed with Leucoperm A following surface staining, and permeabilized with Leucoperm B before adding antibodies for intracellular labelling. The intracellular cytokine production by non-stimulated DC was measured using superenhanced D_{max} (SED) normalised subtraction (see below) to subtract the normal cumulative histogram for cytokine staining with no added monensin from a similar histogram of staining with cytokine and added monensin for the last 4 hours of cell culture (Sigma, UK). This protocol has been validated by our group [8] and allows us to assess the natural on-going cytokine production (without external PMA and/or ionomycin stimulus) of DC. After incubation cells were washed in FACS buffer, fixed and acquired.

Flow cytometry and data analysis

Cells were acquired on a FACSCalibur cytometer (BD Biosciences) and analysed using WinList 5.0 software (Verity, ME, US). The proportion of cells positive for a given marker was determined by reference to staining with an isotype-matched control antibody. For single parameter analysis, WinList was used to subtract the normal cumulative histogram for isotype control staining from a similar histogram of staining with the test antibody using the superenhanced D_{max} (SED) normalised subtraction [53]. For multiple parameter analysis positive and negative "gates" were set up, determined by reference to staining with isotype-matched control antibodies.

Proliferation assays

T-cells were obtained from freshly isolated PBMC, and suspended in MiniMACs buffer (PBS containing 0.5% BSA and 2mM EDTA). PBMC were depleted of CD14, CD19 and HLA-DR positive cells with immunomagnetic beads (Miltenyi Biotech, Bisley, UK) following manufacturer's instructions. An average of 94.91%±1.06 (mean±SD) viable T-cells were obtained following enrichment. T-cells were labelled with CFSE (Invitrogen Ltd, UK) following manufacturer's instructions. Initial CFSE-labelled T-cells (400,000) were incubated for 5 days in round-bottomed 96 well microtitre plates (Becton Dickinson) with or without different concentrations of allogeneic DC (1%, 2% or 3% of T-cells), previously conditioned in a different microenvironment. Cells were recovered and percentage and phenotype of stimulated T-cells (CFSE^{low}) were quantified by flow cytometry within total CD3+ cells in the lymphogate. Optimization experiments confirmed that best proliferation rates with allogeneic CFSE-labelled T-cells were obtained at day 5 with no proliferated T-cells before day 4 and with over 80% of the cells undergoing cell division after day 7 –data not shown-.

Statistical analyses

Two-tailed paired t-test, two-tailed Pearson´s correlation and one- or two-way paired ANOVA were applied as stated in the figure legends. In the case of multiple

comparisons, subsequent *ad-hoc* Bonferroni correction was applied. The level of significance was fixed at $p < 0.05$.

CONFLICT OF INTEREST

No authors had financial conflict of interest that might be construed to influence the results or interpretation of their manuscript.

REFERENCES

[1] Mowat, A.M., Anatomical basis of tolerance and immunity to intestinal antigens. Nat Rev Immunol. 2003. 3: 331-41.

[2] Heller, F., Florian, P., Bojarski, C., Richter, J., Christ, M., Hillenbrand, B., Mankertz, J,, et al. Interleukin-13 is the key effector Th2 cytokine in ulcerative colitis that affects epithelial tight junctions, apoptosis, and cell restitution. Gastroenterology. 2005. 129: 550-64.

[3] Strober, W. and Fuss, I.J., Proinflammatory cytokines in the pathogenesis of inflammatory bowel diseases. Gastroenterology. 2011. 140: 1756-67.

[4] Larsen, S., Bendtzen, K. and Nielsen, O.H., Extraintestinal manifestations of inflammatory bowel disease: epidemiology, diagnosis, and management. Ann Med. 2010. 42: 97-114.

[5] Veloso, F.T., Extraintestinal manifestations of inflammatory bowel disease: Do they influence treatment and outcome? World J Gastroenterol. 2011. 17: 2702-7.

[6] Banchereau, J. and Steinman, R.M., Dendritic cells and the control of immunity. Nature. 1998. 392: 245-52.

[7] Niess, J.H. and Reinecker, H.C. Dendritic cells: the commanders-in-chief of mucosal immune defenses. Curr Opin Gastroenterol. 2006. 22: 354-60.

[8] Hart, A.L., Al-Hassi, H.O., Rigby, R.J., Bell, S.J., Emmanuel, A.V., Knight, S.C., Kamm, M.A. and Stagg, A.J., Characteristics of intestinal dendritic cells in inflammatory bowel diseases. Gastroenterology. 2005. 129: 50-65.

[9] Chirdo, F.G., Millington, O.R., Beacock-Sharp, H. and Mowat, A.M., Immunomodulatory dendritic cells in intestinal lamina propria. Eur J Immunol. 2005. 35: 1831-40.

[10] Coombes, J.L., Siddiqui, K.R., Arancibia-Cárcamo, C.V., Hall, J., Sun, C.M., Belkaid, Y. and Powrie F., A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. J Exp Med. 2007. 204: 1757-64.

[11] Stagg, A.J., Kamm, M.A. and Knight, S.C., Intestinal dendritic cells increase T cell expression of alpha4beta7 integrin. Eur J Immunol. 2002. 32: 1445-54.

[12] von Boehmer, H., Oral tolerance: is it all retinoic acid? J Exp Med. 2007. 204: 1737-9.

[13] Sigmundsdottir, H. and Butcher, E.C., Environmental cues, dendritic cells and the programming of tissue-selective lymphocyte trafficking. Nat Immunol. 2008. 9: 981-7.

[14] Mora, J.R., Iwata, M., von Andrian, U.H., Vitamin effects on the immune system: vitamins A and D take centre stage. Nat Rev Immunol. 2008. 8: 685-98.

[15] Mora, J.R., Iwata, M., Eksteen, B., Song, S.Y., Junt, T., Senman, B., Otipoby, K.L., et al., Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. Science. 2006. 314: 1157-60.

[16] Iwata, M., Hirakiyama, A., Eshima, Y., Kagechika, H., Kato, C. and Song, S.Y., Retinoic acid imprints gut-homing specificity on T cells. Immunity. 2004. 21: 527-38.

[17] Mora, J.R. and von Andrian, U.H., Role of retinoic acid in the imprinting of gut-homing IgA-secreting cells. Semin Immunol. 2009. 21: 28-35.

[18] Hadis, U., Wahl, B., Schulz, O., Hardtke-Wolenski, M., Schippers, A., Wagner, N., Müller, W., et al., Intestinal tolerance requires gut homing and expansion of FoxP3+ regulatory T cells in the lamina propria. Immunity. 2011. 34: 237-46.

[19] La Cava, A., Van Kaer, L. and Fu-Dong-Shi. CD4+CD25+ Tregs and NKT cells: regulators regulating regulators. Trends Immunol. 2006. 27: 322-7.

[20] Seino, K. and Taniguchi, M., Functionally distinct NKT cell subsets and subtypes. J Exp Med 2005. 202: 1623-6.

[21] Kim, H.J., Hwang, S.J., Kim, B.K., Jung, K.C. and Chung, D.H., NKT cells play critical roles in the induction of oral tolerance by inducing regulatory T cells producing IL-10 and transforming growth factor beta, and by clonally deleting antigen-specific T cells. Immunology 2006. 118: 101-11.

[22] Rimoldi, M., Chieppa, M., Salucci, V., Avogadri, F., Sonzogni, A., Sampietro, G.M., Nespoli, A., et al., Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells. Nat Immunol. 2005. 6: 507-14.

[23] Butler, M., Ng, C.Y., van Heel, D.A., Lombardi, G., Lechler, R., Playford, R.J. and Ghosh, S., Modulation of dendritic cell phenotype and function in an in vitro model of the intestinal epithelium. Eur J Immunol.2006. 36: 864-74.

[24] Iliev, I.D., Mileti, E., Matteoli, G., Chieppa, M. and Rescigno, M., Intestinal epithelial cells promote colitis-protective regulatory T-cell differentiation through dendritic cell conditioning. Mucosal Immunol. 2009. 2: 340-50.

[25] Iliev, I.D., Spadoni, I., Mileti, E., Matteoli, G., Sonzogni, A., Sampietro, G.M., Foschi, D., et al., Human intestinal epithelial cells promote the differentiation of tolerogenic dendritic cells. Gut. 2009. 58: 1481-9.

[26] Mann, E.R., Bernardo, D., Al-Hassi, H.O., English, N.R., Clark, S.K., McCarthy, N.E., Milestone, A.N., et al. In humans, gut-specific homeostatic dendritic cells are generated from blood precursors by the gut microenvironment. Inflamm Bowel Dis. 2011. doi: 10.1002/ibd.21893.

[27] León, A.J., Gómez, E., Garrote, J.A., Bernardo, D., Barrera, A., Marcos, J.L., Fernández-Salazar, L., et al., High levels of proinflammatory cytokines, but not markers of tissue injury, in unaffected intestinal areas from patients with IBD. Mediators Inflamm.2009. 2009:580450.

[28] Dudda, J.C., Lembo, A., Bachtanian, E., Huehn, J., Siewert, C., Hamann, A., Kremmer, E., et al., Dendritic cells govern induction and reprogramming of polarized tissue-selective homing receptor patterns of T cells: important roles for soluble factors and tissue microenvironments. Eur J Immunol. 2005. 35: 1056-65.

[29] Huff, K.R., Akhtar, L.N., Fox, A.L., Cannon, J.A., Smith, P.D. and Smythies, L.E.,

Extracellular matrix-associated cytokines regulate CD4+ effector T-cell responses in the human intestinal mucosa. Mucosal Immunol. 2011. 4: 420-7.

[30] Bogunovic, M.; Ginhoux, F.; Helft, J.; Shang, L.; Hashimoto, D.; Greter, M.; Liu, K.; et al., Origin of the lamina propria dendritic cell network. Immunity. 2009. 31:513-25.

[31] Varol, C.; Vallon-Eberhard, A.; Elinav, E.; Aychek, T.; Shapira, Y.; Luche, H.; Fehling, H.J.; et al., Intestinal lamina propria dendritic cell subsets have different origin and functions. Immunity. 2009. 31:502-12.

[32] Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions.
Schulz, O.; Jaensson, E.; Persson, E.K.; Liu, X.; Worbs, T.; Agace, W.W.; et al. J Exp Med. 2009. 206:3101-14.

[33] Laffont, S.; Siddiqui, K.R. and Powrie, F., Intestinal inflammation abrogates the tolerogenic properties of MLN CD103+ dendritic cells. Eur J Immunol. 2010. 40:1877-83.

[34] Aymeric, R.; Jianping, H.; Abhisake, K.; Vassilis, V.; and Brian, L.K., Inflammation switches the differentiation program of Ly6C^{hi} monocytes from antiinflammatory macrophages to inflammatory dendritic cells in the colon. J Exp Med. doi: 10.1084/jem.20101387

[35] DePaolo, R.W., Abadie, V., Tang, F., Fehlner-Peach, H., Hall, J.A., Wang, W., Marietta, E.V., et al., Co-adjuvant effects of retinoic acid and IL-15 induce inflammatory immunity to dietary antigens. Nature. 2011. 471: 220-4.

[36] Bernardo, D., van Hoogstraten, I.M., Verbeek, W.H., Peña, A.S., Mearin, M.L., Arranz, E., Garrote, J.A., et al., Decreased circulating iNKT cell numbers in refractory coeliac disease. Clin Immunol. 2008. 126: 172-9.

[37] Arihiro, S., Ohtani, H., Suzuki, M., Murata, M., Ejima, C., Oki, M., Kinouchi, Y., et al., Differential expression of mucosal addressin cell adhesion molecule-1 (MAdCAM-1) in ulcerative colitis and Crohn's disease. Pathol Int. 2002. 52: 367-74.

[38] Hart, A.L., Kamm, M.A., Knight, S.C. and Stagg, A.J., Quantitative and functional characteristics of intestinal-homing memory T cells: analysis of Crohn's disease patients and healthy controls. Clin Exp Immunol. 2004. 135: 137-45.

[39] Hart, A.L., Kamm, M.A., Knight, S.C. and Stagg, A.J. Prospective evaluation of intestinal homing memory T cells in ulcerative colitis. Inflamm Bowel Dis. 2004. 10: 496-503.

[40] Kawashima, R., Kawamura, Y.I., Oshio, T., Son, A., Yamazaki, M., Hagiwara, T., Okada, T., et al., Interleukin-13 Damages Intestinal Mucosa via

TWEAK and Fn14 in Mice-A Pathway Associated With Ulcerative Colitis. Gastroenterology. 2011. 141: 2119-2129.

[41] Gibbons, D.L. and Spencer, J., Mouse and human intestinal immunity: same ballpark, different players; different rules, same score. Mucosal Immunol. 2011. 4: 148-57.

[42] Inoue, S., Matsumoto, T., Iida, M., Mizuno, M., Kuroki, F., Hoshika, K. and Shimizu, M., Characterization of cytokine expression in the rectal mucosa of ulcerative colitis: correlation with disease activity. Am J Gastroenterol. 1999. 94: 2441-6.

[43] Bernardo, D.; Garrote, J.A.; Allegretti, Y.; León, A.; Gómez, E.; Bermejo-Martin, J.F., Calvo, C. et al. Higher constitutive IL15R alpha expression and lower IL-15 response threshold in coeliac disease patients. Clin Exp Immunol. 2008. 154:64-73.

[44] Sanchez-Munoz, F., Dominguez-Lopez, A., Yamamoto-Furusho, J.K., Role of cytokines in inflammatory bowel disease. World J Gastroenterol. 2008. 14: 4280-8.

[45] Li, Y., de Haar, C., Chen, M., Deuring, J., Gerrits, M.M., Smits, R., Xia, B., et al. Disease-related expression of the IL6/STAT3/SOCS3 signalling pathway

in ulcerative colitis and ulcerative colitis-related carcinogenesis. Gut. 2010. 59: 227-35.

[46] Brand, S., Crohn's disease: Th1, Th17 or both? The change of a paradigm: new immunological and genetic insights implicate Th17 cells in the pathogenesis of Crohn's disease. Gut. 2009. 58: 1152-67.

[47] Olsen, T., Rismo, R., Cui, G., Goll, R., Christiansen, I. and Florholmen J., TH1 and TH17 interactions in untreated inflamed mucosa of inflammatory bowel disease, and their potential to mediate the inflammation. Cytokine. 2011 PMID: 21945121

[48] Nishimoto, N. and Kishimoto, T., Humanized antihuman IL-6 receptor antibody, tocilizumab. Handb Exp Pharmacol. 2008. 181: 151-60.

[43] [49] Kitamura, K., Nakamoto, Y., Kaneko, S. and Mukaida, N., Pivotal roles of interleukin-6 in transmural inflammation in murine T cell transfer colitis. J Leukoc Biol. 2004. 76: 1111-7.

[50] Mitsuyama, K., Matsumoto, S., Masuda, J., Yamasakii, H., Kuwaki, K., Takedatsu, H. and Sata, M., Therapeutic strategies for targeting the IL-6/STAT3 cytokine signaling pathway in inflammatory bowel disease. Anticancer Res. 2007. 27: 3749-56.

[51] Holden, N.J., Bedford, P.A., McCarthy, N.E., Marks, N.A., Ind, P.W., Jowsey, I.R., Basketter, D.A. and Knight, S.C., Dendritic cells from control but not atopic donors respond to contact and respiratory sensitizer treatment in vitro with differential cytokine production and altered stimulatory capacity. Clin Exp Allergy. 2008. 38: 1148-59.

[52] Ng, S.C., Plamondon, S., Al-Hassi, H.O., English, N., Gellatly, N., Kamm, M.A., Knight, S.C. and Stagg, A.J., 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: 205-18.

[53] Bagwell, C.B., Hyperlog-a flexible log-like transform for negative, zero, and positive valued data. Cytometry A. 2005. 64: 34-42.

[54] Ren, M., Pozzi, S., Bistulfi, G., Somenzi, G., Rossetti, S. and Sacchi, N., Impaired retinoic acid (RA) signal leads to RARbeta2 epigenetic silencing and RA resistance. Mol Cell Biol. 2005. 25: 10591-603.

[55] Ozbek, S.M., Ozbek, A. and Erdorgan, A.S., Analysis of Enterococcus faecalis in samples from Turkish patients with primary endodontic infections and failed endodontic treatment by real-time PCR SYBR green method. J Appl Oral Sci.2009. 17: 370-4.

[56] Lundgren, A., Strömberg, E., Sjöling, A., Lindholm, C., Enarsson, K., Edebo, A., Johnsson, E., et al., Mucosal FOXP3-expressing CD4+ CD25high regulatory T cells in Helicobacter pylori-infected patients. Infect Immun. 2005. 73: 523-31.

[57] Ferreyra Solari, N.E., Galoppo, C., Cuarterolo, M., Goñi, J., Fernández-Salazar, L., Arranz, L.E., Garrote, J.A. and Cherñavsky, A.C., The simultaneous high expression of Vα24, IFN-γ and FoxP3 characterizes the liver of children with type I autoimmune hepatitis. Clin Immunol. 2010. 137: 396-405.

FIGURE LEGENDS

FIGURE 1: inflamed areas from UC biopsies have increased pro-inflammatory cytokines secretion.
A) Secretion of soluble cytokines, following 24 hours culture of colonic biopsies, from inflamed affected areas of UC patients and paired biopsies from healthy unaffected areas from the same patients. **B)** Cytokine profile in inflamed areas from UC patients, and **C)** correlation between secreted IL-6 from inflamed biopsies and Mayo endoscopic score. Paired t-test and Pearson´s correlation were applied respectively. P-value below 0.05 was considered statistically significant (*p<0.05, **p<0.01).

FIGURE 2: IL-13 blockade has no relevant effect in the cytokine profile of inflamed areas from UC patients.
Biopsies from inflamed areas of UC patients were cultured with and without 2.5µg/ml of blocking anti IL-13 and the secretion of soluble cytokines, following 24 hours culture, was assayed. Paired t-test was applied. P-value below 0.05 was considered statistically significant (*p<0.05, **p<0.01).

FIGURE 3: IL-13 supplementation in healthy areas from UC patients does not promote a TH2 microenvironment.
Biopsies from non-inflamed or healthy areas of UC patients were cultured with and without 50ng/ml of recombinant IL-13 and the secretion of soluble cytokines, following 24 hours culture, was assayed. Paired t-test was applied. P-value below 0.05 was considered statistically significant (***p<0.001).

FIGURE 4: IL-6 blockade in inflamed areas from UC patients decreases the pro-inflammatory microenvironment.
Biopsies from inflamed areas of UC patients were cultured with and without 0.5µg/ml of blocking anti IL-6 and the secretion of soluble cytokines, following 24 hours culture was assayed. Paired t-test was applied. P-value below 0.05 was considered statistically significant (**p<0.01, ***p<0.001).

FIGURE 5: IL-6 supplementation in healthy areas from UC patients promotes a pro-inflammatory microenvironment.
Biopsies from non-inflamed or healthy areas of UC patients were cultured with and without 50ng/ml of recombinant IL-6 and the secretion of soluble cytokines, following

24 hours culture assayed. Paired t-test was applied. P-value below 0.05 was considered statistically significant (*p<0.05, **p<0.01, ***p<0.0001).

FIGURE 6: mRNA profile of healthy and inflamed colonic biopsies from UC patients.

mRNA expression, in arbitrary units (U) referred to housekeeping GADPH, in 24 hours cultured colonic biopsies from inflamed affected areas of UC patients and paired biopsies from healthy unaffected areas, of 16s, MUC2, FOXP3, Vα24, CX3CR1 and RALDH2. Paired t-test was applied. P-value below 0.05 was considered statistically significant (*p<0.05).

FIGURE 7: Inflamed areas from UC biopsies activate DC

A) HLA-DR and CD40 intensity ratio (IR) and percentage of CCR7, CX3CR1, and CCR8 on blood DC conditioned with colonic biopsy culture supernatant (SN) from healthy and inflamed areas of UC patients compared to unconditioned (basal) DC. Shaded area represents positive events after subtraction from isotype histograms. Each histograms is representative of several independent experiments (HLA-DR, n=12; CD40, n=10; CX3CR1, n=12; CCR7, n=11; CCR8, n=7). **B)** Pool of experiments displaying mean±SEM. One way ANOVA repeated measures and paired t-test following Bonferroni correction were applied. P-value below 0.05 was considered statistically significant (*p<0.05, **p<0.01).

FIGURE 8: DC conditioned with inflamed areas from UC patients fail to acquire a regulatory cytokine profile.

A) Ongoing production of IL-10, IL-12 (p40/p70), IL-6 and TGFβ in DC following conditioning with biopsy culture supernatants (SN) from healthy and inflamed areas of UC patients compared to unconditioned (basal) DC. Shaded area represents the percentage of positive cells following subtraction from paired DC which had been incubated in the absence of monensin. Histograms are representative of several independent experiments (IL-10, n=11; IL12(p40/p70), n=12; IL-6, n=12; TGFβ, n=12). **B)** Pool of experiments displaying mean±SEM. One-way ANOVA repeated measures and paired t-test following Bonferroni correction were applied. P-value below 0.05 was considered statistically significant (*p<0.05).

FIGURE 9: DC conditioned with inflamed areas from UC patients increased their stimulatory capacity and prime T-cells with skin-homing capacity

A) Percentage of proliferating CFSE-labelled T-cells following 5 days culture alone (resting) or in the presence of 3% allogeneic DC which had been previously conditioned with culture supernatants (SN) from healthy and inflamed areas from UC patients or unconditioned (basal) DC and **B)** pool of 10 independent paired experiments displaying mean±SEM. T-cell proliferative responses were both dependent on the doses of DC (p<0.001) and their previous conditioning (p<0.001). Healthy-SN conditioned DC were less stimulatory that basal (p<0.01 at 3%) and inflamed-conditioned DC (p<0.05 at 2% and p<0.001 at 3%) with no differences among the latter. **C)** β7 (gut-homing) and CLA (skin-homing) dot plots on resting T-cells (unconditioned) and T-cells stimulated (CFSE^{low}) by 3% basal, healthy-SN or inflamed-SN DC and **D)** pooled data after several independent experiments. Healthy and inflamed areas from the same patients where used to condition DC from the same healthy donor in all cases. Paired t-test was applied. P-value below 0.05 was considered statistically significant (*p<0.05, **p<0.01).

FIGURE 10: IL-6 mediates the increased stimulatory and skin-homing imprinting capacity of DC in inflamed areas of UC patients.

A) Stimulatory capacity of DC on allogeneic T-cells correlates with their IL-6 ongoing production. **B)** and **C)**, stimulatory capacity of DC on T-cells was dependent on the percentage of DC (p<0.001) and their previous conditioning (p<0.001). **B)** DC conditioned with inflamed SN which had been blocked for IL-6 were less stimulatory that their unblocked counterparts at 3% (p<0.001, n=10). **C)** DC conditioned with healthy areas from UC patients were less stimulatory than their unconditioned (basal) counterparts (p<0.05, n=4). However, if healthy areas had been stimulated with IL-6, conditioned DC did not decreased their stimulatory capacity (p<0.01, n=4). **D)** Pooled data after several independent experiments displaying CLA expression on stimulated T-cells following culture with stimulated DC. Percentage of divided T-cells and phenotype of stimulated T-cells were determined as stated in Figure 9. Pearson´s correlation test, two-way ANOVA repeated measures and paired t-test were applied. P-value below 0.05 was considered statistically significant (*p<0.05, ***p<0.001).

Table 1: clinical data of UC patients enrolled in this study.

Patient	Gender	Age	Mayo endoscopic Score	Extension
1	male	54	2	3
2	male	29	2	3
3	male	34	3	2
4	female	64	3	3
5	female	51	1	1
6	male	70	1	2
7	female	57	3	1
8	female	65	1	1
9	female	82	2	2
10	female	50	1	2
11	female	66	2	1
12	male	66	1	2

Data includes gender, age, Mayo endoscopic score (from 0 to 3) and extension of the disease (1: only rectal; 2: lesion affects up to the splenic flexure; 3: lesion passes the splenic flexure).

Table 2: quantitative PCR primers

Molecule	Primers sequence	Ta	Primers source
GADPH	Fw 5'-GAAGGTGAAGGTCGGAGTC-3'	60	[54]
	Rv 5'-GAAGATGGTGATGGGATTTC-3'		
16s	Fw 5'-TTAAACTCAAAGGAATTGACGG-3'	68	[55]
	Rv 5'-CTCACGRCACGAGCTGACGAC-3'		
FoxP3	Fw 5'-CAGCACATTCCCAGAGTTCCTC-3'	68	[56]
	Rv 5'-CGCTGTGAACCAGTGGTAGATC-3'		
Vα24	Fw 5'-CTGGAGGGAAAGAACTGC-3'	55	[57]
	Rv 5'-TGTCAGGGAAACAGGACC-3'		
MUC2	-	60	Hs00159374_Applied
CX3CR1	-	60	5532957001_Roche
RALDH2	-	60	5532957001_Roche

Primers used for quantitative PCR including primer sequence, annealing temperature (Ta) and primers source.

Table 3: Antibodies and flow cytometry

Antibody Specificity	Clone	Conjugate	Manufacturer
HLA-DR	L243 (G46-6)	PECy5	BD Biosciences
CD40	LOB7/6	FITC	AbD Seroter
CD83	HB15e	PE	BD Biosciences
CD86	BU63	PE	AbD Serotec
CX3CR1	528728	PE	R&D
TLR2	TLR2.3	FITC	AbD Seroter
TLR4	HTA125	FITC	AbD Seroter
β7	FIB504	PE	BD Biosciences
β7	FIB504	PECy5	BD Biosciences
CLA	HECA-452	FITC	BD Biosciences
CLA	HECA-452	Biotin	BD Biosciences
Streptavidin	-	APC	BD Biosciences
CD103	Ber-ACT8	FITC	BD Biosciences
CCR4	205410	APC	R&D
CCR10	314315	APC	R&D
CCR7	150503	PE	R&D
IL-6	AS12	FITC	BD Biosciences
IL-10	JES3-19F1	APC	BD Biosciences
IL-12(p40/p70)	C11.5	PE	BD Biosciences
TGFβ	35409	PE	R&D
CD3	UCHT1	PeCy5	BD Biosciences

Specificity, clone, conjugated fluorochrome and manufacturer of the monoclonal antibodies used.
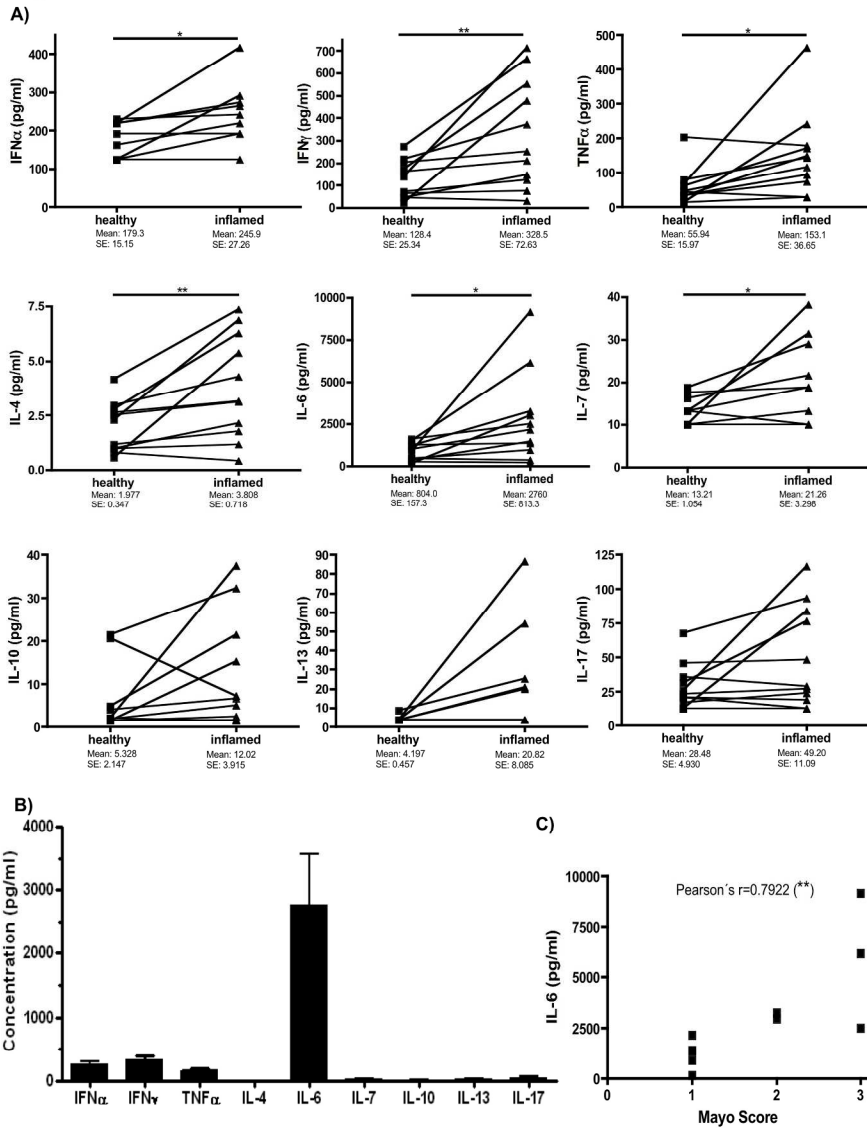
Figure 1

793x1057mm (72 x 72 DPI)

Figure 2

793x1057mm (72 x 72 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
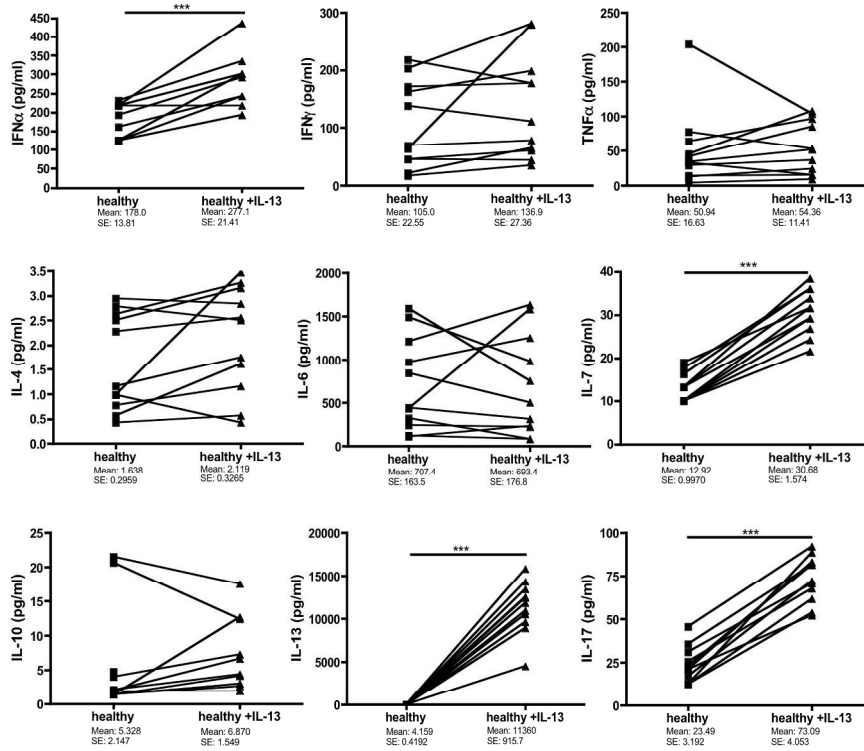46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
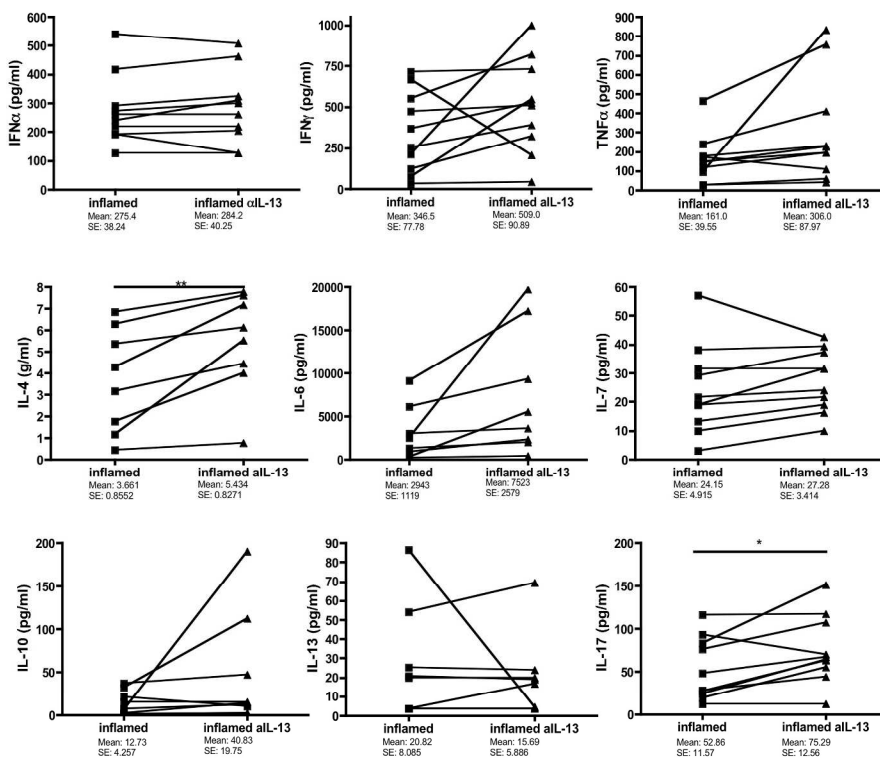
Figure 3

793x1057mm (72 x 72 DPI)

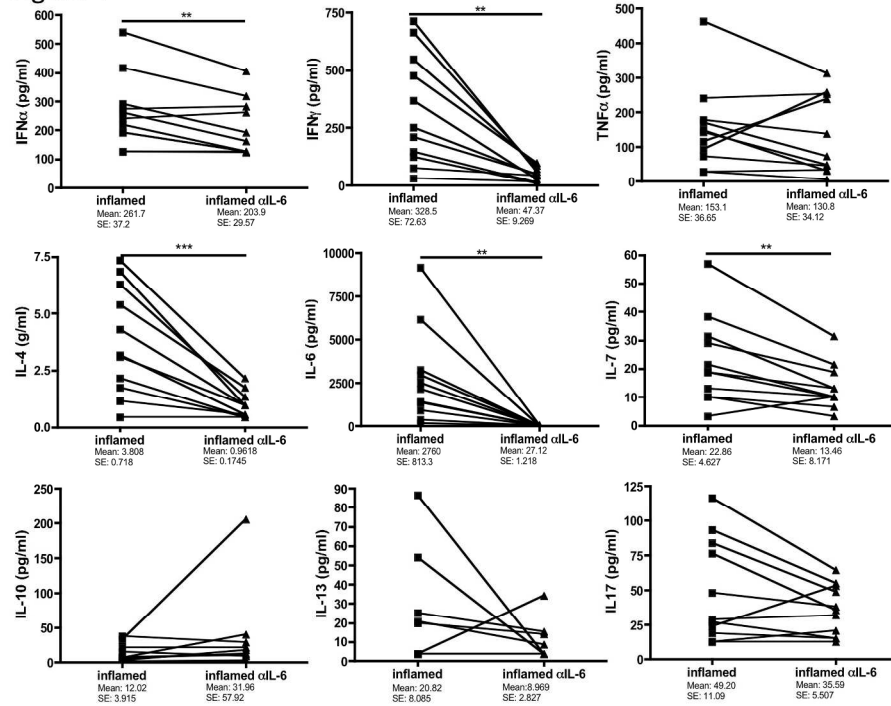Figure 4

793x1057mm (72 x 72 DPI)

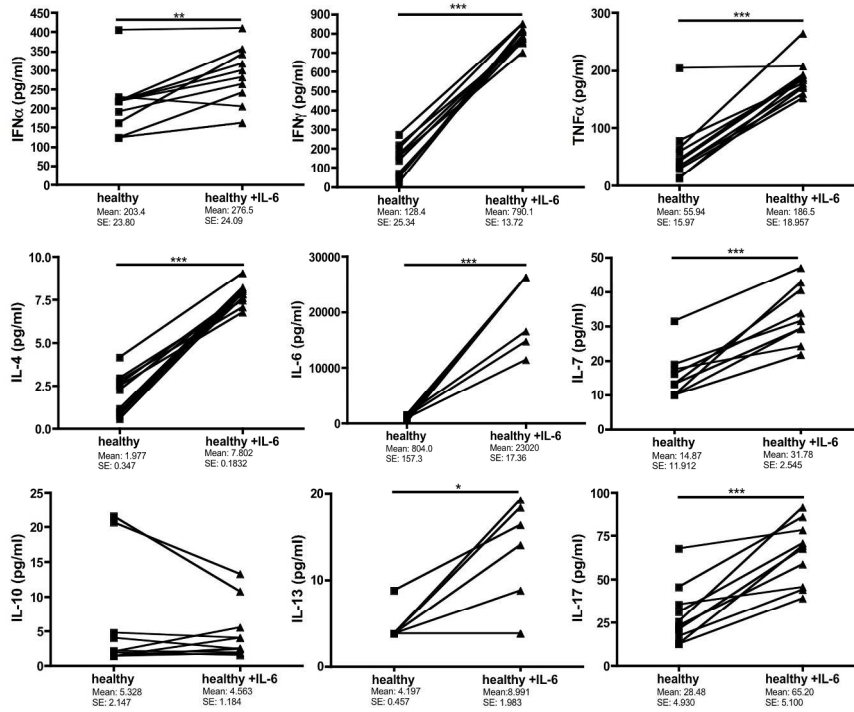1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
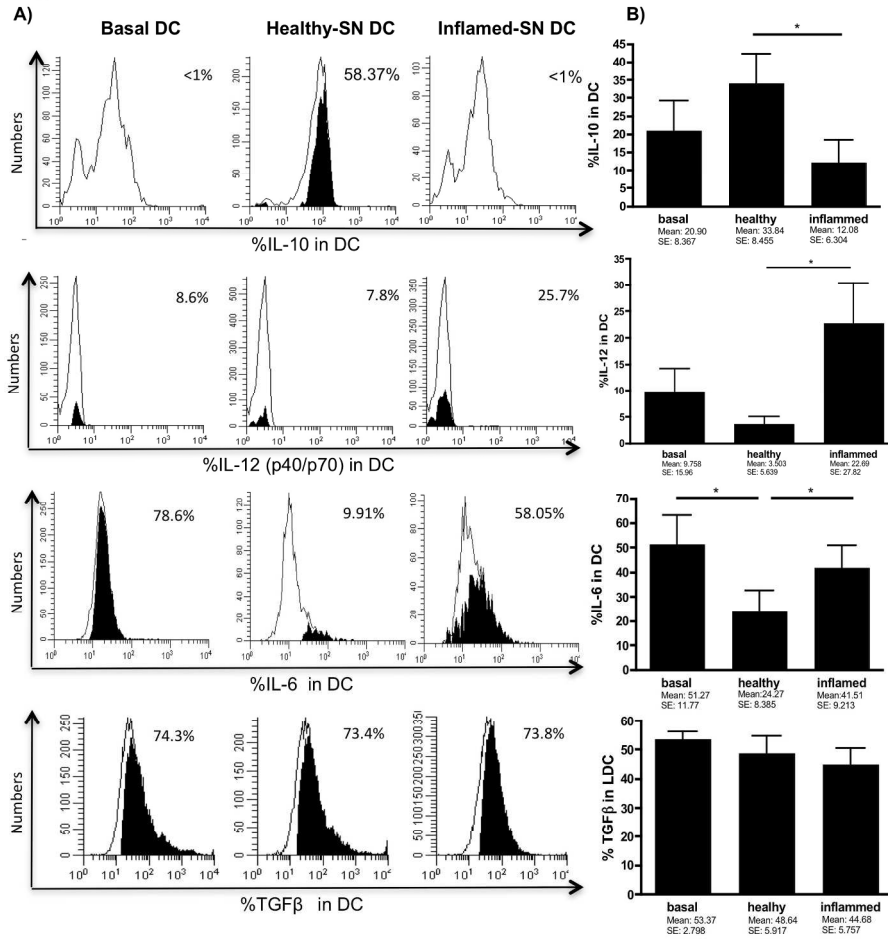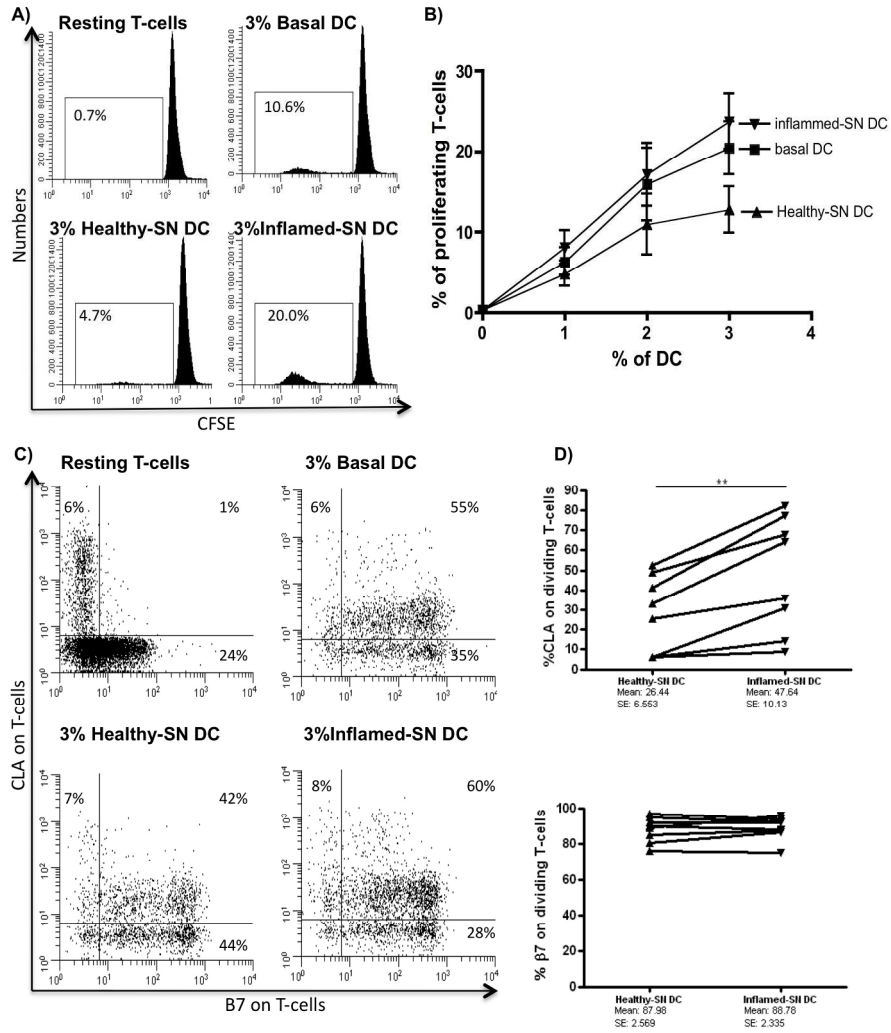58
59
60

Figure 5

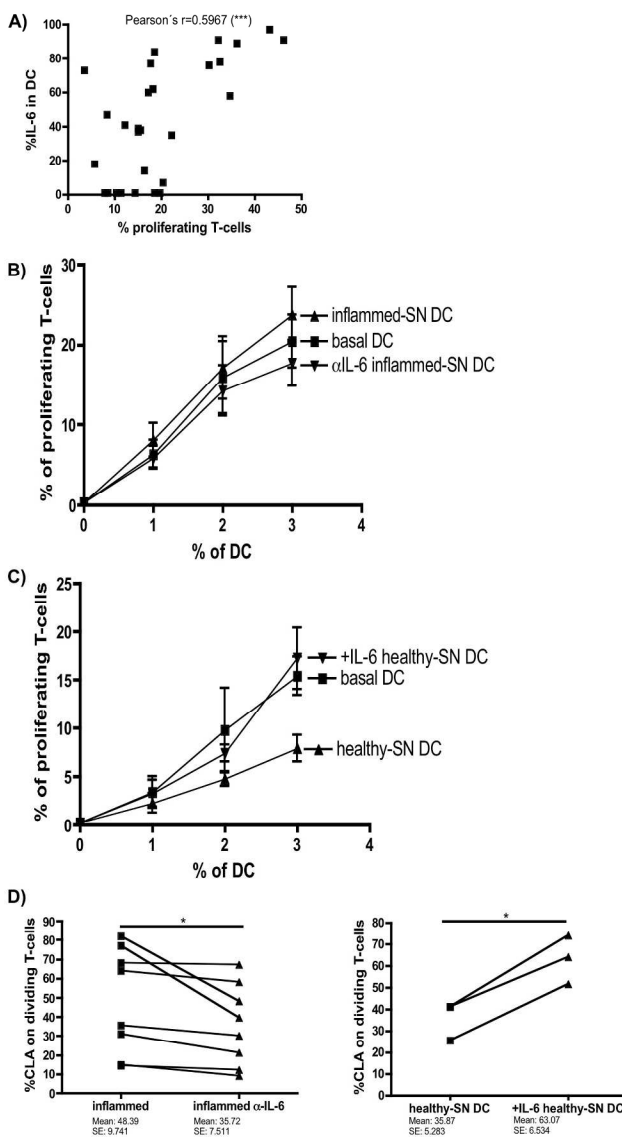793x1057mm (72 x 72 DPI)

Figure 6
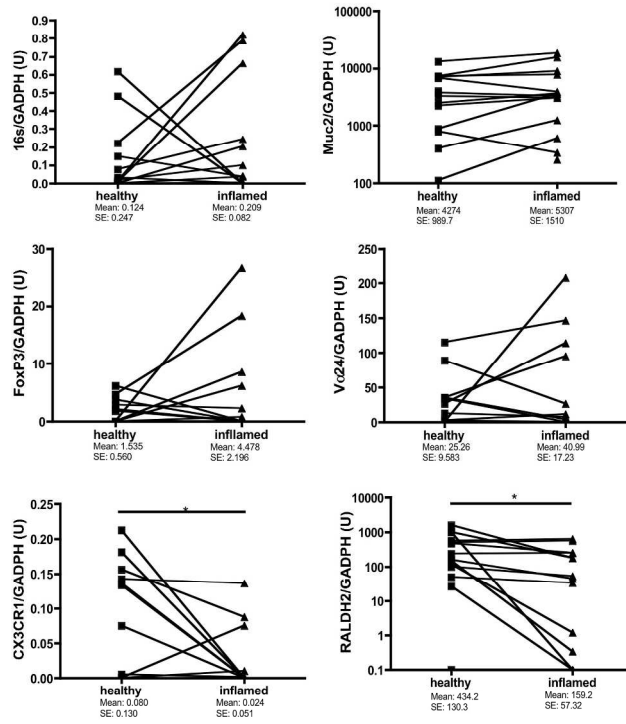
793x1057mm (72 x 72 DPI)

Figure 7
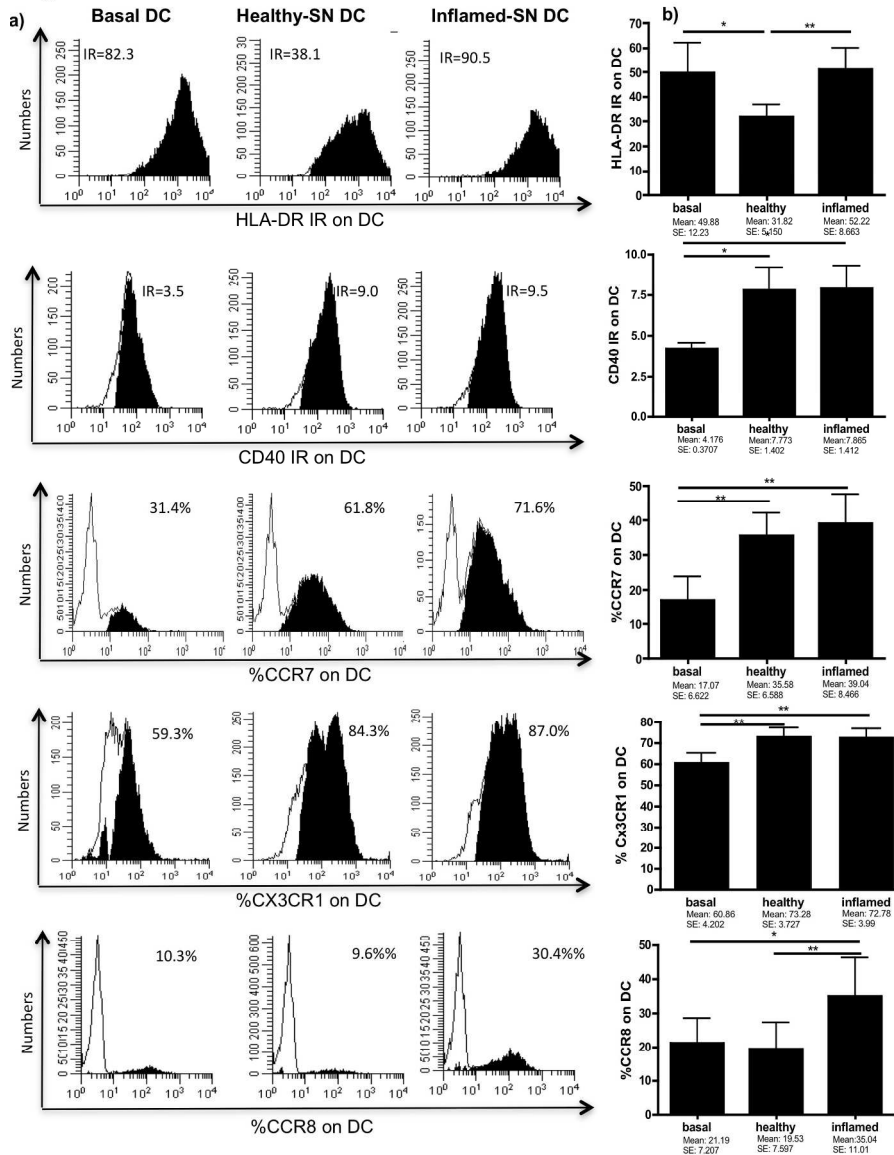
793x1057mm (72 x 72 DPI)

Figure 8

793x1057mm (72 x 72 DPI)

Figure 9

793x1057mm (72 x 72 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Figure 10

793x1057mm (72 x 72 DPI)