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Article Title : M1 Macrophages Activate Notch Signalling in Epithelial Cells: Relevance in Crohn's Disease

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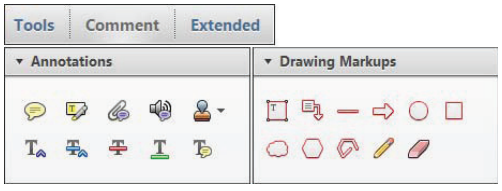

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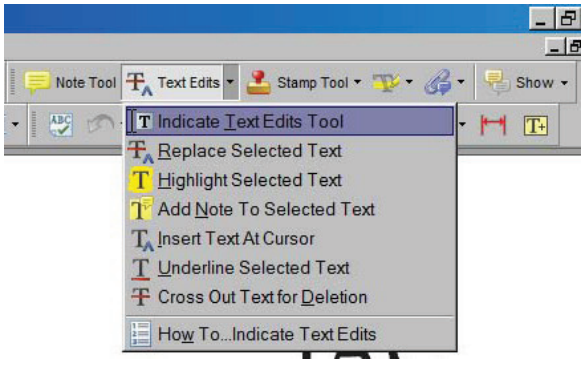

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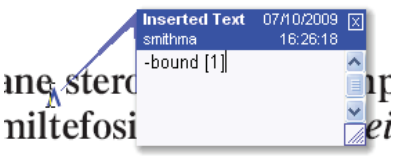

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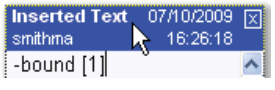

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M1 Macrophages Activate Notch Signalling in Epithelial Cells: Relevance in Crohn's Disease

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Abstract

Background: The Notch signalling pathway plays an essential role in mucosal regeneration, which constitutes a key goal of Crohn's disease (CD) treatment. Macrophages coordinate tissue repair and several phenotypes have been reported which differ in the expression of surface proteins, cytokines and hypoxia-inducible factors (HIFs). We analysed the role of HIFs in the expression of Notch ligands in macrophages and the relevance of this pathway in mucosal regeneration.

Methods: Human monocytes and U937-derived macrophages were polarized towards the M1 and M2 phenotypes and the expression levels of HIF-1α, HIF-2α, jagged 1 (Jag1) and delta-like 4 (Dll4) were evaluated. The effects of macrophages on the expression of HES1 (the main target of Notch signalling) and IAP (enterocyte marker) in epithelial cells in co-culture were also analysed. Phenotype macrophage markers and Notch signalling were evaluated in the mucosa of CD patients.

Results: M1 macrophages were associated with HIF-1-dependent induction of Jag1 and Dll4, which increased HES1 protein levels and IAP activity in co-cultured epithelial cells. In the mucosa of CD patients a high percentage of M1 macrophages expressed both HIF-1α and Jag1 while M2 macrophages mainly expressed HIF-2α and we detected a good correlation between the ratio of M1/M2 macrophages and both HES1 and IAP protein levels.

Conclusion: M1, but not M2, macrophages are associated with HIF-1-dependent induction of Notch ligands and activation of epithelial Notch signalling pathway. In the mucosa of chronic CD patients, the prevalence of M2 macrophages is associated with diminution of Notch signalling and impaired enterocyte differentiation.

Key Words: Macrophages; Crohn's disease; mucosal healing; Notch signalling

1. Introduction

Crohn's disease (CD) is a chronic relapsing inflammatory disorder of the gastrointestinal tract characterized by transmural inflammation, architectural distortion and thickening of all the layers of the bowel wall, which leads to intestinal fibrosis and stricture development.[1] The aim of current clinical management is to prolong periods of remission and halt the destructive and progressive course of the disease. In recent years mucosal healing has been established as a key treatment goal in CD that predicts sustained clinical remission and resection-free survival of patients.[2,3] This process is highly dependent on the adequate reconstruction of the intestinal epithelium, which depends on proliferation and differentiation of the progenitor cells located at the base of the crypts. The coordination of several

signalling pathways, including Wnt and Notch, plays an essential role in epithelial regeneration.[4–10]

The Notch signalling pathway is mediated by Notch proteins, which act as receptors for the transmembrane ligands Jagged (Jag) and Delta-like (Dll) proteins. Upon binding to their ligands, Notch receptors are cleaved by γ-secretase and the Notch intracellular domain translocates to the nucleus, where it up-regulates the expression of specific target genes, such as *HES1*. This gene, in turn, represses the expression of Math1, a master regulator of secretory cell lineage differentiation.[6,7] It was initially reported that deletion of the *HES1* gene resulted in the generation of an excessive number of secretory cells.[11] Later studies demonstrated that inactivation of Notch signalling results in conversion of proliferating progenitors into post-mitotic goblet cells,[12] which led to the assumption that Notch signalling plays an essential role in regulating cell-fate decisions in intestinal homeostasis. However, there is still controversy regarding the regulation of Notch signalling in CD. An increase in *Math1* mRNA expression has been reported in the damaged mucosa of CD patients[13] while increased cleavage of Notch-1, which is the upstream signal regulating HES1 expression, has also been described.[14]

Macrophages constitute one of the central components of the inflamed mucosa, where local hypoxia and inflammatory mediators modulate their gene expression through the activity of hypoxia-inducible factors (HIFs).[15,16] Several macrophage phenotypes have been characterized, and differ in the expression of surface proteins and the production of cytokines.[17] The M1 or pro-inflammatory phenotype mediates the defence of the host from microorganisms and contributes to inflammatory injury. There is evidence in the literature of a role for the transcription factor HIF-1 in M1 polarization,[18] and several studies report the up-regulation of Notch receptors and Notch signalling in classical macrophage differentiation.[19–21] The M2 macrophage phenotype expresses high levels of anti-inflammatory cytokines and coordinates tissue repair.[22,23] It has recently been reported that inhibition of Notch signalling enhances M2 polarization.[20] In the present study we analysed the role of HIF in the expression of Notch ligands in macrophages. In addition, taking into account the strategic position of macrophages in maintaining communication with epithelial cells, we explored the relevance of macrophages in the regulation of Notch signalling and regeneration of the mucosa of CD patients.

2. Materials and methods

2.1. Intestinal mucosal samples

Colonic surgical resections were obtained from the damaged mucosa of CD patients and from the healthy mucosa of patients with colorectal cancer (as controls) (Table 1). The study was approved by the Institutional Review Board of The Hospital of Manises (Valencia). Written informed consent was obtained from all participating patients.

Table 1. Patient characteristics.

Patient group and characteristic	*n*
Crohn's disease	16
Age (y)	
17–40	6
>40	10
Gender	
Male	9
Female	7
Concomitant medication	
Azathioprine	3
Anti-tumour necrosis factor	16
Mesalazine	2
Control	11
Age (y)	
17–40	0
>40	11
Gender	
Male	7
Female	4

2.2. Isolation of colonic crypts

Human intestinal crypts were isolated from the mucosa of surgical resections obtained from control and CD patients, as described previously.[24]

2.3. Isolation of macrophages from human intestine

Macrophages were isolated from the mucosa of surgical resections obtained from control and CD patients as described previously.[24]

2.4. Cell culture

Caco-2 cells (American Type Culture Collection, VA) were cultured in MEM medium (Sigma-Aldrich) supplemented with 20% inactivated foetal bovine serum (FBS) with 100 Um/L penicillin, 100 mg/mL streptomycin, 2 mM l-glutamine (Lonza), 100 mM sodium pyruvate (Lonza) and 1% non-essential amino acids (Lonza).

HT29 cells (American Type Culture Collection, VA, USA) were cultured in McCoy's Medium Modified (Sigma-Aldrich) supplemented with 10% inactivated FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM l-glutamine.

Human monocytes (U937, European Collection of Cell Culture, Salisbury, UK) were cultured in RPMI medium with 10% inactivated FBS with 100 U/mL penicillin and 100 µg/mL streptomycin. Monocytes were differentiated into macrophages by culturing them in the presence of phorbol myristate acetate (PMA) for 48 h.[25] U937-derived macrophages were stimulated with lipopolysaccharide (LPS; 0.1 µg/mL; *E. coli* 0111:B4) and interferon γ (IFN-γ; 20 ng/mL) or with interleukin 4 (IL-4; 20 ng/mL) in order to polarize them towards M1 or M2 phenotypes, respectively, as previously reported.[26]

Hypoxia (3% O_2) was established by incubating macrophages in a CO_2/O_2 incubator (Invivo2 400, Ruskinn Technology Ltd, Pencoed, UK) with a blend of 5% CO_2 and the appropriate percentages of O_2 and N_2 to a total of 100%. Normoxic controls were obtained by incubating the cells at 21% O_2.

2.5. Isolation of mononuclear cells

Human peripheral blood mononuclear cells were isolated from both healthy donors and CD patients by Ficoll density-gradient centrifugation at 400*g* for 40 minutes. Monocyte-derived macrophages were obtained from monocytes seeded in 12-well tissue culture plates and differentiated into macrophages by culture in X-Vivo 15 medium (Lonza) supplemented with 1% human serum, 100 U/mL penicillin, 100 µg/mL streptomycin and 20 ng/mL recombinant human macrophage colony-stimulating factor (M-CSF, Peprotech, London, UK) at 37°C in 5% CO_2 for 6 days.

2.6. Co-culture

U937-derived macrophages were seeded and differentiated as above. Afterwards the epithelial cells were placed in the same wells at a ratio 1:1 and were maintained in co-culture for 24 hours.

2.7. Alkaline phosphatase activity

Following 24 hours of co-culture with macrophages, cells were washed with cold phosphate-buffered saline (PBS) and lysed in

150 µL of 0.5% Triton X-100, 10 mM Tris–HCl (pH 8) and 150 mM NaCl. Each sample was mixed with a *p*-nitrophenyl phosphate solution (Sigma-Aldrich). Thirty minutes later, absorbance at 405 nm was measured. Protein content was quantified using the Bradford assay (Bio-Rad Laboratories, Madrid, Spain). Alkaline phosphatase activity was also determined in macrophages cultured alone.

2.8. Immunohistochemistry, immunofluorescence and goblet cell count

AQ11

Immunohistochemistry for HES1, CD68, CD86 and CD206 cells was performed in 5 µm sections of paraffin-embedded tissues (Table 2). A horse anti-mouse/rabbit biotinylated antibody (Vector Laboratories, CA, USA, 1:200) was used as a secondary antibody as previously described.[26] An area of 0.3 mm^2 was selected for quantitative analysis.

Goblet cells were counted following standard periodic acid–Schiff staining of the sections adjacent to those used for immunostaining. We counted the number of goblet cells (by counting the vacuoles) in at least three crypts per sample and results were normalized to the total number of epithelial cells (by counting the nuclei) in the same crypt.

2.9. Static cytometry

AQ16

Macrophages isolated from the mucosa of surgical resections obtained from control and CD patients were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton-X100 and double-stained (Jag1/CD86, Jag1/CD206, HIF1α/CD86, HIF1α/CD206, HIF2α/CD86, HIF2α/CD206, CD86/CD68, CD206/CD68, arginase I/CD68, iNOs/CD68) with specific monoclonal antibodies (Table 2) as previously described.[24] Fluorescence-labelled (TR and FITC) goat anti-mouse or goat anti-rabbit (1:100, Santa Cruz Biotechnology) was used as the secondary antibody, and Hoechst 33342 was added to stain the nuclei.

U937 macrophages co-cultured with epithelial cells were incubated overnight at 4°C with monoclonal antibodies against HES1 or Muc2 combined with an antibody against CD18 to identify and exclude macrophages from the cytometric analysis (Table 2). Fluorescence-labelled (TR and FITC) goat anti-mouse or goat anti-rabbit (1:100, Santa Cruz Biotechnology) were used as the secondary antibodies, and Hoechst 33342 was added to stain the nuclei. In all cases the fluorescent signal (16 images per well) was quantified using the static cytometer software Scan® version 2.03.2 (Olympus, Barcelona, Spain).

2.10. RNA interference and cellular transfection

U937 cells were transfected with a vector targeting human HIF-1α (*miHIF-1α*, described previously[27]) or a non-targeting control vector (*mock*), as described previously.[27] In addition, we have now designed vectors targeting human Jag1 (*miJag1*; 28.82 ± 15.70% of reduction vs *mock*, based on the targeting sequence 5′-CCTAAGCATGGGTCTTGCAAA-3′; GenBank accession number NM_000214.2) and Dll4 (*miDll4*; 27.74 ± 13.39% of reduction vs *mock*, based on the targeting sequence 5′-TCCAACTGCCCTTCAATTTCA-3′; GenBank accession number NM_019074.3). Lipofectamine-2000 (Invitrogen Life Technologies, Carlsbad, CA) was employed as a transfection reagent according to the manufacturer's instructions. Twenty-four hours post-transfection, cells were incubated for 8 h in normoxic or hypoxic conditions, as described above. M1 macrophages were transfected with *miHIF-1α*, *miJag1*, *miDll4* or a *mock* vector before M1 polarization.

2.11. Protein extraction and Western blot analysis

Equal amounts of protein from macrophages, HT29 cells, Caco-2 cells or colonic tissue[28] were loaded onto sodium dodecyl sulphate/polyacrylamide gel electrophoresis gels and analysed by Western blot as described previously (Table 2). Protein expression was quantified by means of densitometry using Image Gauge Version 4.0 software (Fujifilm). Data were normalized to β-actin.

2.12. RNA extraction and quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analysis

Total RNA and cDNA from macrophages or colonic tissue was obtained as described previously.[25] Real-time PCR was performed with the PrimeScript Reagent Kit Perfect Real Time (Takara) in a thermocycler (LightCycler, Roche Diagnostics). Specific oligonucleotides were designed according to the sequences shown in Table 3.

2.13. Chromatin immunoprecipitation (ChIP) assay

A ChIP assay was carried out in U937-derived macrophages, incubated under hypoxia or normoxia for 5 h, as previously described.[25] Immunoprecipitation was performed with anti-HIF1α antibody (BD, Madrid, Spain) or control IgG antibody. After reverse crosslinking, DNA fragments were purified with a Montage PCR Kit (Millipore, Germany). PCR was performed

Table 2. Specific antibodies used for immunohistochemistry, immunofluorescence studies and Western blot analysis.

AQ12

Antibody	Immunofluorescence	Immunohistochemistry	Western blot
	Antibody dilution	Antigen retrieval	Antibody dilution
IAP (Santa Cruz Biotechnology)			1:1000
HES1 (Santa Cruz Biotechnology)	1:100	Sodium citrate buffer pH 6°C, 20 min, 1:200	1:500
AQ14 Jag1 (Santa Cruz Biotechnology)	1:100	Sodium citrate buffer pH 9°C, 20 min, 1:200	
CD18 (BD, Barcelona Spain)	1:100		
CD68 (Biolegend, Madrid, Spain)	1:100	α-Chymotrypsin 37°C, 20 min, 1:100	
CD86 (Epitomics, Burlingame, CA, USA)	1:100	α-Chymotrypsin 37°C, 20 min, 1:200	
AQ15 CD206 (Novus Biologicals, Cambridge, UK)	1:100	Sodium citrate buffer pH 9°C, 20 min, 1:200	
Arginase I (Santa Cruz Biotechnology)	1:100		
iNOs (Santa Cruz Biotechnology)	1:100		
HIF-1α (Novus Biologicals)	1:100		1:500
HIF-2α (Santa Cruz Biotechnology)	1:100		
Muc2 (Santa Cruz Biotechnology)	1:100		
β-actin (Sigma-Aldrich)			1:10 000

AQ13

Table 3. Primer sequences of specific PCR products for each gene analysed.

Human gene	Sense	Antisense	Length (bp)
Jag1	5′-gaacacgggcgttgcccact-3′	5′-gtggacgcatcccgggtgtg-3′	304
Dll4	5′-gtgcagcgtacaccggcact-3′	5′- tctgttcgcgacgccgcttt-3′	223
HES1	5′-aaaattcctcgtccccggtg-3′	5′-tttgt tatccgttcg-3′	64
Muc2	5′-gctggccgccggctattacc-3′	5′-accccggccgtcatccatca-3′	79
Math1	5′-ccgcccagtatttgctacat-3′	5′-cattcacctgtttgctggaa-3′	234
β-actin	5′-ggacttcgagcaagagatgg-3′	5′-agcactgtgttggcgtacag-3′	67

using PCR Master (Roche Diagnostics, Mannheim, Germany) with the primers 5′-TGTCCACCCTTCAAAGGAAGTC-3′ and 5′-CAAATCCGAGTCTGCGGAGC-3′, detecting the region −1646 to −1166 in the *Jag1* promoter, or 5′-CCCTGAGCATCCCGCTG-3′ and 5′-CCGGCTCTAATATACTCCGCC-3′, detecting the region −638 to −106 in the *Jag1* promoter, as shown in Figure 1c. The PCR products were separated by electrophoresis in 2% agarose gel.

2.14. Statistical analysis

Data were expressed as mean ± SEM and compared by one-way analysis of variance (ANOVA) with Newman–Keuls *post hoc* correction for multiple comparisons or a *t*-test when appropriate. A p value <0.05 was considered to be statistically significant. Clinical correlations were analysed in the human samples using Pearson's correlation coefficient.

3. Results

3.1. HIF-1 mediates the expression of Notch ligands in M1 macrophages

AQ17

Hypoxia induced a time-dependent increase in HIF-1α stabilization in macrophages, which peaked at 8 hours and then progressively decreased. In parallel, hypoxia induced a time-dependent increase in the mRNA expression of *Jag1* and *Dll4* compared with the expression detected in cells in normoxia (Figure 1a). To evaluate the role of HIF-1 in gene expression, we used an miRNA approach to selectively knockdown this transcription factor in U937-derived macrophages. As shown in Figure 1b, up-regulation of the mRNA expression of both *Dll4* and *Jag1* induced by hypoxia was significantly reduced in cells transfected with *miHIF1α*, showing that HIF-1 is involved in the induction of these ligands in hypoxia.

AQ18

Analysis of the Jag1 gene promoter identified potential HIF-1 binding sites (HRE sequence). To examine the binding of HIF-1α to the promoter region of Jag1, we performed ChIP assays using an affinity-purified antibody directed against HIF-1α and primers specific for two Jag1 promoter regions containing HIF-1 binding sites (Figure 1c). Our data revealed HIF-1α binding to the proximal promoter region of the *Jag1* gene in hypoxia through the HRE sequences located between positions −106 and −638 (Figure 1c) from the start codon.

Polarization of U937 macrophages towards an M1 phenotype[26] following treatment with LPS + IFN induced HIF-1α stabilization within the first 24 hours and failed to induce HIF-2α stabilization significantly at any time analysed (Figure 2a). In contrast, polarization towards an M2 phenotype[26] as a result of treatment with IL-4 induced HIF-2α stabilization but not HIF-1α stabilization (Figure 2a). Analysis of the expression of HIF-1 target genes revealed a significant increase (fold induction) in the mRNA expression of *LDHA* (2.2 ± 0.4) and *iNOS* (6.0 ± 1.7) by M1 macrophages compared with both non-polarized (1.0 ± 0.2 and 0.9 ± 0.1, respectively) and M2 (1.2 ± 0.3 and 2.4 ± 1.3, respectively) macrophages. In addition, the expression of an HIF-2 target gene, *ArgI*, was increased in M2 macrophages (2.7 ± 0.7) compared with non-polarized (1.0 ± 0.1) and M1 (0.8 ± 0.1) cells. Analysis of the expression of Notch ligands revealed a significant increase in mRNA expression of *Dll4* and mRNA expression and protein levels of *Jag1* in M1 macrophages but not in M2 cells (Figure 2b). These effects were also observed in macrophages derived from primary monocytes obtained from both healthy donors and CD patients (Figure 2c). The up-regulation of the mRNA expression of both *Dll4* and *Jag1* that was detected in U-937 macrophages polarized towards an M1 phenotype was significantly reduced in cells transfected with *miHIF1α* (Figure 2d), demonstrating that HIF-1 is involved in the induction of Notch ligands in M1 macrophages.

3.2. HIF-1-dependent induction of Notch ligands mediates the increase in HES1 expression and IAP activity induced by M1 macrophages

Next we analysed whether macrophages modulate the Notch signalling pathway and markers of differentiation in co-cultured epithelial cells. First, we determined the expression of HES1 and IAP (a marker of enterocyte differentiation) protein levels in two epithelial cell lines, HT29 and Caco-2, at sub-confluence and at different times after reaching cell confluence. Our data show a time-dependent increase in protein levels of both HES1 and IAP as well as IAP enzymatic activity in both HT29 and Caco-2 cells by culturing post-confluence (Supplementary Figure 1).

In the co-culture experiments, M1 macrophages increased protein levels of HES1 and IAP enzymatic activity with no effect on Muc2 expression in either HT29 or Caco-2 cells (Figure 3a–d). The effects induced by M1 macrophages on epithelial protein levels of HES1 were significantly reduced in macrophages treated with *miHIF1*, *miDLL4* and *miJag1*, suggesting that the HIF-1-dependent induction of Notch ligands mediates the activation of Notch signalling in epithelial cells (Figure 3a, c). In contrast, M2 macrophages did not significantly modify HES1 protein levels but induced a significant reduction in IAP activity in both HT29 and Caco-2 cells (Figure 3a–d). No IAP activity was detected in macrophages.

3.3. M1 macrophages express HIF-1α and Notch ligands while M2 macrophages express HIF-2α in the mucosa of CD patients

Next we performed a comparative study of control and CD patients to characterize the macrophage phenotype present in the mucosa of CD patients and the expression of HIF and Notch ligands in these cells.

Immunohistochemical experiments revealed macrophages in an adjacent position to epithelial cells (Figure 4a) and a quantitative analysis showed that the numbers of CD68+ cells and CD206+ cells were significantly higher in the mucosa of chronic CD patients than

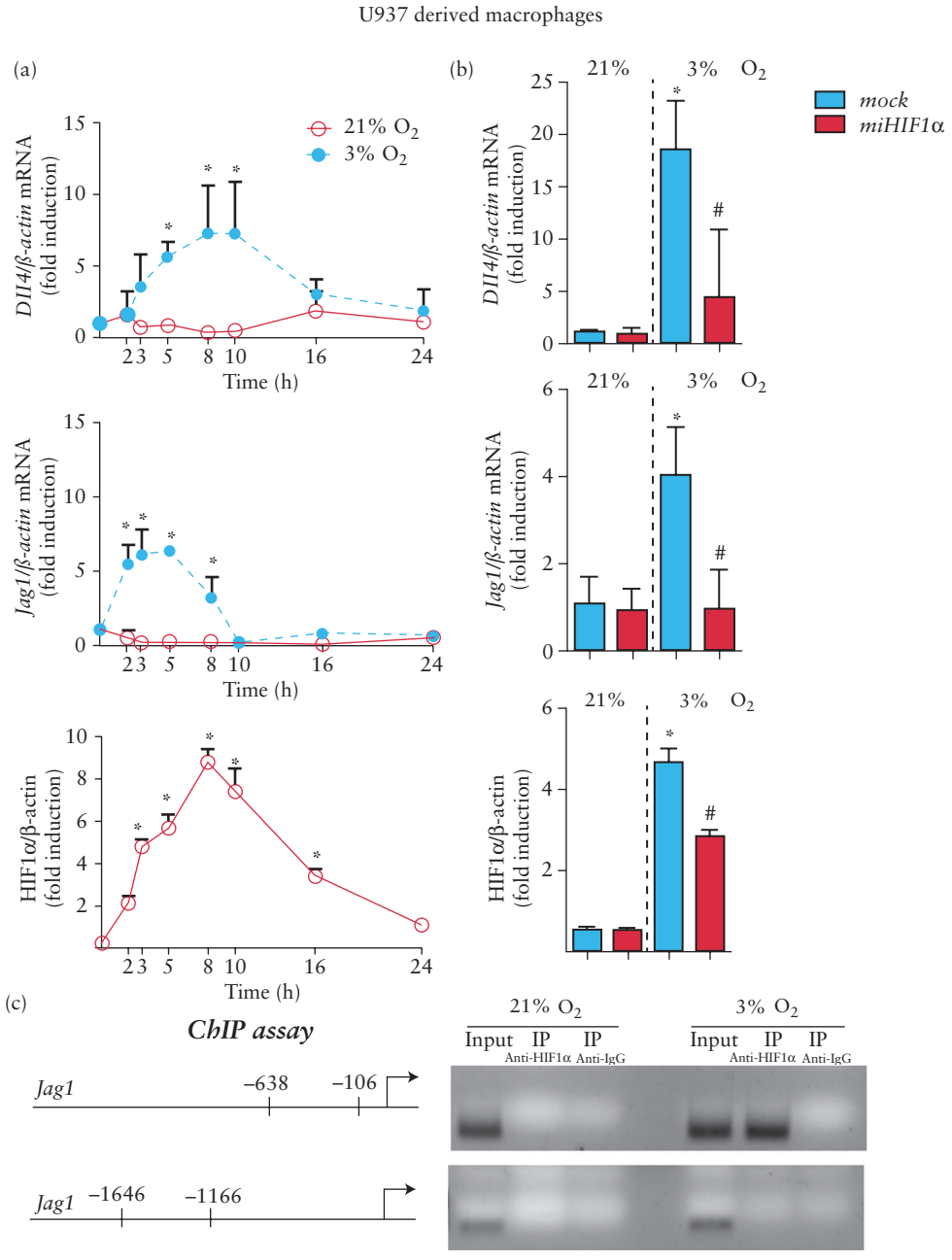

Figure 1. HIF-1 mediates the hypoxic up-regulation of Dll4 and Jag1 in macrophages. (a) Graphs showing time-course analysis of the effects of hypoxia on HIF-1α protein levels and mRNA expression of Jag1 and Dll4 in U937 macrophages. In all cases, points in the graphs represent mean ± SEM ($n > 3$). *$p < 0.05$ vs time 0 h or vs macrophages in normoxia at the same time point. (b) Graphs showing mRNA expression of Dll4 and Jag1 and protein levels of HIF-1α in mock-transfected U937 cells and cells treated with *miHIF-1α* under normoxic or hypoxic conditions (8 h). In all cases, bars in the graphs represent mean ± SEM ($n > 3$). *$p < 0.05$ vs the same group in normoxia; #$p < 0.05$ vs *mock*-transfected cells in hypoxia. (c) Representative chromatin immunoprecipitation (ChIP) experiment performed in samples from U937-derived macrophages in normoxia or hypoxia. Chromatin was immunoprecipitated with anti-HIF-1α antibody or a non-related antibody anti-IgG as a control. An aliquot of the input chromatin is also shown. Primers specific to the promoter region of the *Jag1* gene were used to amplify the DNA isolated from the ChIP assay.

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in that of control patients. In contrast, no significant differences were observed in the number of CD86+ cells (Figure 4b). Double immunofluorescence experiments in macrophages isolated from the mucosa revealed that the percentage of CD68-positive cells that expressed M1 markers CD86 and iNOS was similar in CD and control patients. In contrast, the percentage of CD68-positive cells that expressed M2 markers CD206 and ArgI was significantly higher in macrophages isolated from the mucosa of CD patients (Figure 4c).

We also detected a high percentage of CD86+ cells expressing HIF-1α in macrophages isolated from both control and CD patients, while a very low percentage of CD206+ cells expressed HIF-1α (Figure 5b). In contrast, a large percentage of CD206+ cells from the mucosa of both control and CD patients expressed HIF-2α while a low percentage of CD86+ cells expressed HIF-2α (Figure 5c). Finally, the percentage of cells expressing the Notch ligand Jag1 in the population of CD86+ cells was higher than that recorded in cells

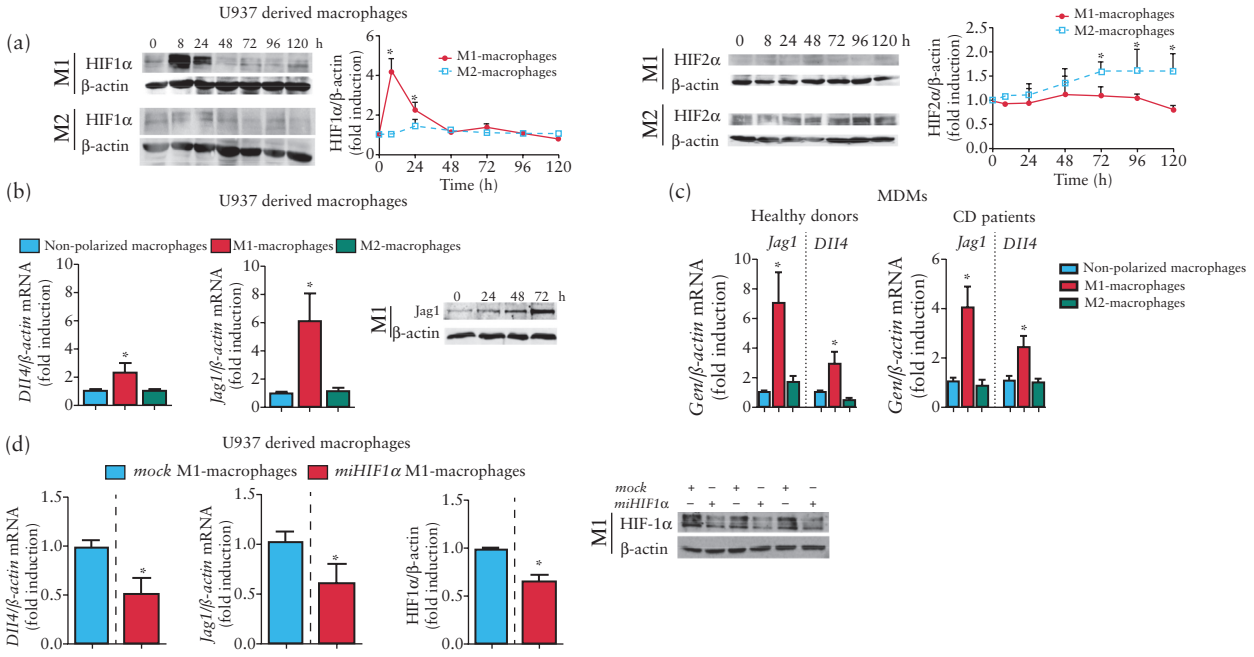

Figure 2. HIF-1 mediates the expression of Notch ligands associated with M1 macrophages. U937-derived macrophages (n = 6) were either treated with lipopolysaccharide (LPS) and interferon-γ (IFN-γ) and polarized towards M1 macrophages or treated with interleukin 4 (IL-4) and polarized towards M2 macrophages; some cells were treated with the vehicle (non-polarized macrophages). (a) Representative Western blots and graph showing HIF-1α or HIF-2α protein levels at different time points. Points in the graphs represent mean ± SEM (n > 3). *p < 0.05 vs time 0 h. (b) Graphs show relative mRNA expression levels of *Dll4* and *Jag1* in macrophages and protein levels of Jag1 in M1 macrophages at different time points. Bars represent mean ± SEM. *p < 0.05 vs the respective value in non-polarized macrophages and M2 macrophages. (c). Graphs showing relative mRNA expression levels of *Dll4* and *Jag1* in macrophages derived from primary monocytes (healthy donors, n = 6; Crohn's disease patients, n = 6). Bars represent mean ± SEM. *p < 0.05 vs the respective value in non-polarized macrophages and M2 macrophages. (d) Representative Western blots and graphs showing protein levels of HIF-1α and mRNA expression of *Dll4* and *Jag1* in U937-derived macrophages transfected with *mock* or *miHIF-1*α and polarized towards M1. Bars represent mean ± SEM. *p < 0.05 vs *mock* M1 macrophages.

expressing CD206 (Figure 5a) and these values were similar between macrophages obtained from control and CD patients (Figure 5a).

3.4. Macrophages modulate Notch signalling and markers of differentiation in human intestine in a phenotype-dependent manner

To determine whether macrophages in the mucosa modulate Notch signalling in epithelial cells, we analysed this pathway specifically in crypts isolated from the mucosa of control and CD patients. Results revealed low HES1 immunostaining and decreased mRNA and protein HES1 expression in the mucosa of CD patients compared with controls (Figure 6a). In addition, we also detected enhanced *Math1* mRNA expression, increased *Muc2* mRNA expression, a higher percentage of goblet cells per crypt and decreased IAP protein levels in the mucosa of chronic CD patients compared with control mucosa, suggesting a diminution of the Notch signalling pathway and impaired differentiation associated with CD. To study a possible regulatory link between M1 macrophages and Notch signalling, the relationship between HES1 protein levels detected by western blot and the proportion of CD86+/CD68+ macrophages was analysed and a positive correlation coefficient (r = 0.4631, p = 0.045) (Figure 5d) was obtained. A detailed analysis revealed a different distribution of points marked by the presence of CD, and when data were analysed separately the correlation coefficient was closer for both controls (r = 0.89, p = 0.001) and CD patients (r = 0.79, p = 0.001). This suggests that other factors were regulating HES1 in the mucosa (Figure 5d). Interestingly, a better correlation coefficient (r = 0.804, p < 0.001) was obtained between HES1 protein levels and the ratio of CD86+ (M1) to CD206+ (M2) macrophages (Figure 6e). The M1/M2 ratio also exhibited a positive and significant correlation with IAP protein levels (r = 0.66, p = 0.002) (Figure 6e), which suggests that both M1 and M2 macrophages regulate Notch signalling in the mucosa.

4. Discussion

The present study demonstrates that M1 macrophages, but not M2 macrophages, are associated with HIF-1-dependent induction of Jag1 and Dll4, which increases HES1 protein levels and IAP activity in co-cultured epithelial cells. In the mucosa of chronic CD patients, the M1/M2 macrophage ratio closely correlates with Notch signalling and markers of enterocyte differentiation, suggesting that macrophages play a role in the diminished Notch signalling and impaired enterocyte differentiation observed.

Our data show that HIF-1, a transcription factor induced by hypoxia and inflammatory conditions, mediates the expression of *Dll4* and *Jag1* in hypoxic macrophages. A previous study reported activation of the Dll4 promoter by HIF-1 in endothelial cells.[29] We demonstrate for the first time the activation of the Jag1 promoter by HIF-1 and provide further evidence that HIF-1 regulates the expression of Notch ligands. Emerging evidence suggests that the functional phenotype of macrophages is regulated by transcription factors that define alternative activation.[18] We found HIF-1α stabilization in human macrophages polarized towards an M1 phenotype and HIF-2α stabilization in those that had been polarized towards an M2 phenotype. Of interest, our data associate for the first time

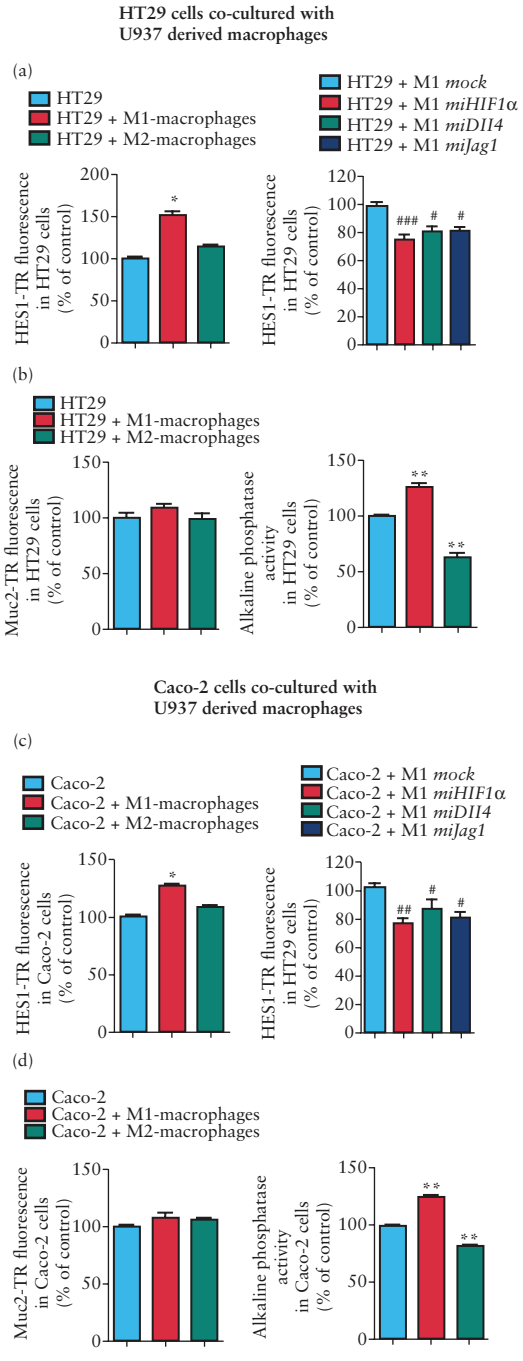

Figure 3. M1 macrophages activate HES1 expression and markers of differentiation in epithelial cells. HT29 cells (a) or Caco-2 cells (b) at pre-confluence were co-cultured (24 h) with M1 or M2 macrophages (stained with CD18 and fluorescein isothiocyanate). In some cases macrophages were transfected with *mock, miHIF1α, miDll4* or *miJag1* vectors previous M1 polarization. Levels of HES1 staining (TR) or Muc2 staining (TR) in epithelial cells were determined by static cytometry ($n = 6$). Graphs show a significant increase in the expression of epithelial HES1 induced by M1 but not M2 macrophages compared with that detected in epithelial cells cultured alone. M1- *miHIF1α*, M1- *miDll4*, M1- *miJag1* macrophages significantly reduced HES1 expression in either HT29 or Caco-2 cells. M1 or M2 macrophages failed to significantly modify the expression of Muc2 in either HT29 or Caco-2 cells. IAP enzymatic activity in epithelial cells was significantly increased by M1 macrophages and significantly reduced by M2 macrophages. In ars represent mean ± SEM. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs epithelial cells; $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ vs epithelial cells co-cultured with M1-*mock* macrophages.

M1, but not M2, macrophages with HIF-1-dependent increases in the mRNA expression of *Dll4* and *Jag1*, which suggests that this transcription factor mediates the selective Notch ligand expression that characterizes the macrophage phenotype. These effects have also been observed in macrophages derived from primary monocytes obtained from both healthy subjects and CD patients properly polarized, supporting the idea of preferential expression of Notch ligands by the M1 phenotype. The pattern of Notch ligand expression is functionally relevant since M1, and not M2, macrophages increased the expression of the main target gene of the canonical Notch signalling, *HES1*, in epithelial cells in co-culture through an action mediated by the HIF-1-dependent induction of Jag1 and Dll4. This was observed in two epithelial cell lines capable of expressing differentiation features characteristic of mature intestinal cells, such as enterocytes or mucus cells.[30, 31] In line with this, the increase in HES1 induced by M1 macrophages was paralleled by an increase in IAP activity, a well-known marker of enterocyte differentiation,[32] with no changes in Muc2 expression. Considering that our results show that spontaneous differentiation of these cells is associated with a time-dependent increase in both IAP activity and HES1 protein level, our results strongly suggest that M1 macrophages promote enterocyte differentiation in epithelial cells. Previous studies have shown that Jag1 up-regulates alkaline phosphatase in stem cells,[33] which leads us to propose that M1 macrophages activate the Notch signalling pathway and enterocyte differentiation in epithelial cells through the expression of Dll4 and Jag1.

We analysed the pathophysiological relevance of these observations in the mucosa of CD patients, in which we found an increased number of macrophages compared with that of control patients. The expression of both M1 and M2 markers was detected but, in a similar manner to that previously reported in the mucosa of ulcerative colitis patients,[26] the number of M2 macrophages was higher than the number of M1 macrophages. Of interest, a high percentage of M1 macrophages were positive for HIF-1α and Jag1, reinforcing the observations reported *in vitro* and suggesting that the expression of Notch ligands by M1 macrophages in human intestine is also associated with HIF-1. Of particular interest, macrophages were frequently detected in an adjacent position to epithelial cells and we observed a positive and significant correlation between CD86+ cells and HES1 protein levels in crypts isolated from the mucosa, which strongly supports the idea that M1 macrophages activate Notch signalling pathways in epithelial cells. A detailed analysis of this correlation revealed differences in the distribution of data marked by the presence or absence of CD; a higher number of macrophages and lower protein levels of epithelial HES1 were detected in the mucosa of CD patients compared with control patients. These observations led us to suggest that, in addition to M1 macrophages, HES1 expression was modulated by other factors present in the inflamed mucosa. Considering our data showing that M2 macrophages prevail in the mucosa of CD patients and that most of them express HIF-2α, which has been related to Notch signalling inhibition,[34] the results suggest that M2 macrophages may also be modulating the Notch pathway. Reinforcing this observation, we have previously demonstrated that M2 macrophages activate Wnt signalling in epithelial cells,[26] and this pathway has been widely associated with inhibition of Notch signalling.[35–37] In line with this, our data show a very good correlation between HES1 protein levels and the ratio of M1/M2 macrophages, and we propose that M2 macrophages act in an opposite manner to M1 cells in the modulation of Notch signalling.

The Notch pathway governs the intestinal binary cell-fate decision between the secretory and absorptive cell lineages [38]. Our

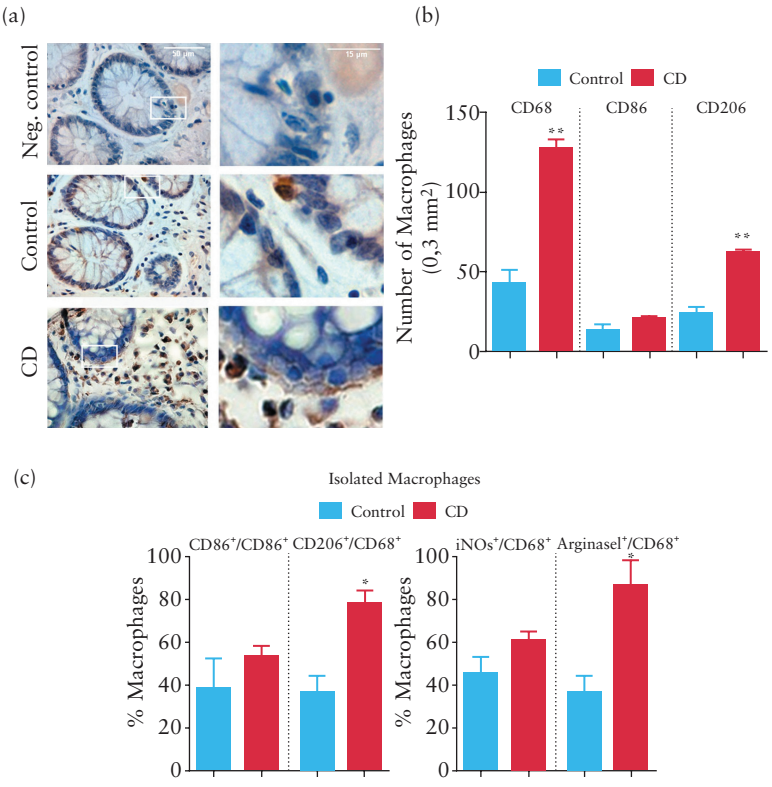

Figure 4. Characterization of the macrophage phenotype in the mucosa of Crohn's disease (CD) patients. (a) Representative images showing CD68 immunostaining in lamina propria and in close proximity to epithelial cells in the mucosa of control and CD patients. (b) Graph showing quantitative analysis of CD68+, CD86+ and CD206+ cells in a total area of 0.3 mm². Bars represent mean ± SEM. $^{**}p < 0.01$ vs control mucosa. (c) Graphs showing the percentages of CD86+/CD68+ cells, CD206+/CD68+ cells, iNOS+/CD68+ cells and arginase I (ArgI)+/CD68+ cells in isolated macrophages obtained from the mucosa (static cytometry). Bars represent mean ± SEM. $^{*}p < 0.01$ vs macrophages from control mucosa.

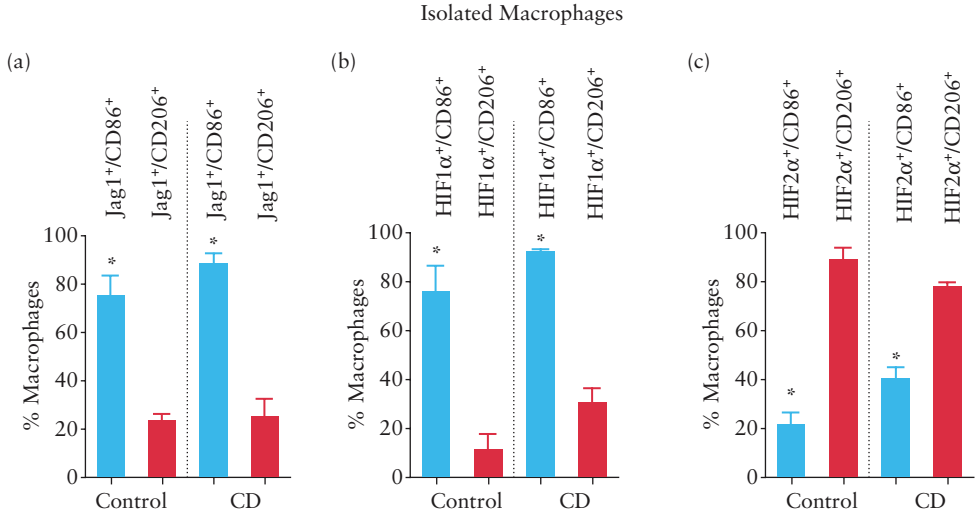

Figure 5. CD86+ macrophages from human mucosa express HIF1α and Jag1. Quantitative analysis (static cytometry) was performed in macrophages isolated from the mucosa and the graph shows the percentages of CD86+ and CD206+ cells that expressed Jag1, HIF-1α or HIF-2α. Bars represent mean ± SEM. $^{*}p < 0.05$ vs CD206+ cells expressing the respective marker in the same group of patients. AQ22

results reveal diminished HES1 expression in crypts isolated from the mucosa of CD patients in parallel with enhanced expression of Math1, a transcription factor that is repressed by HES1, strongly suggesting that the Notch signalling pathway was impaired.[13] It has been reported that the up-regulation of Math 1 directs epithelial cell fate towards secretory lineage cells, including goblet cells.[7, 39] Our data demonstrate increased mRNA expression of *Muc2*, a marker of goblet cells, and a higher number of goblet cells per crypt, in parallel with decreased IAP protein levels in the mucosa of CD patients compared with controls, suggesting that enterocyte differentiation is specifically impaired. Previous studies have reported diminished IAP mRNA and protein expression[40,41] in the intestinal mucosa of adults and children with CD. We extend these observations and show that these diminished IAP protein levels correlate with diminished

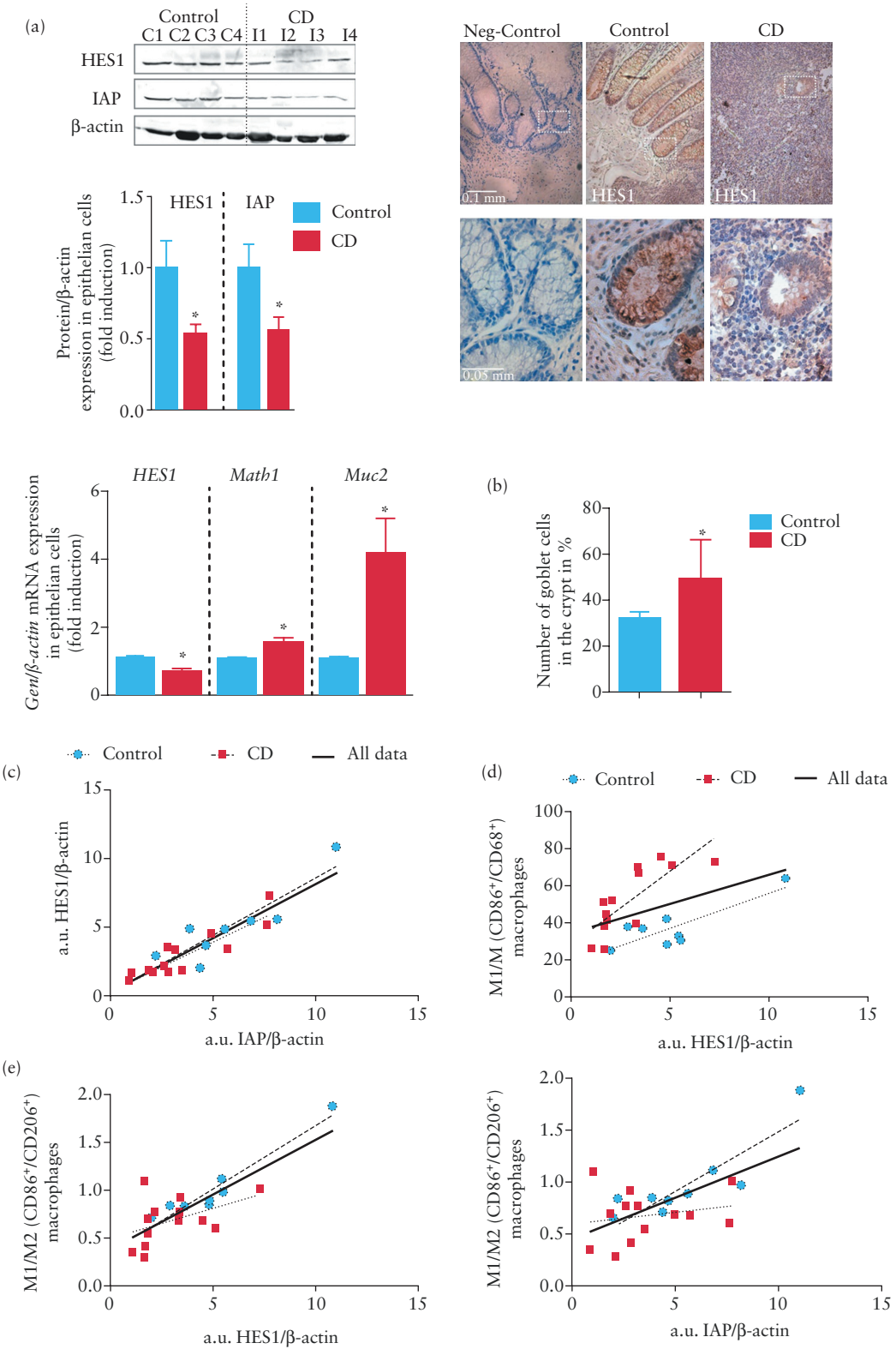

Figure 6. The M1/M2 ratio correlates with HES1 and IAP protein levels in human intestinal mucosa. (a) Representative Western blots showing HES1 and IAP protein levels in the mucosa of Crohn's disease (CD) and control patients and photographs showing HES1 immunostaining. Graphs show quantification of HES1 and IAP protein levels and the mRNA expression of HES1, Math1 and Muc2 in intestinal crypts isolated from the mucosa of control and CD patients. (b) Quantitative analysis showing the percentage of goblet cell vs nuclei per crypt in the mucosa (three crypts were analysed per sample). In all cases bars in graphs represent mean ± SEM. Significant difference from control mucosa is shown by $*p < 0.05$. (c) A positive and significant correlation was observed between HES1 and IAP protein levels in the mucosa of CD and control patients. (d) A positive and significant correlation was observed between CD86+/CD68+ cells and HES1 protein levels in the mucosa of CD and control patients. (e) A closer correlation was established between the CD86+/CD206+ ratio and HES1 protein levels as well as the CD86+/CD206+ ratio and IAP protein levels.

HES1 protein levels, which leads us to propose that enterocyte differentiation is impaired in CD as a consequence of an undermined Notch signalling pathway. This hypothesis is backed by the fact that macrophages, which were closely correlated with HES1 protein levels, were also correlated with the expression of the enterocyte marker IAP.

As a whole, our results provide evidence of HIF-1 dependent induction of Notch ligands associated with M1 macrophages. In contrast to M2 macrophages, M1 cells activate the Notch signalling pathway in epithelial cells. The prevalence of M2 over M1 macrophages in the mucosa of chronic CD patients may mediate the diminished enterocyte differentiation and impaired mucosal regeneration observed in these patients. A better understanding of the reciprocal regulation of macrophage phenotype and mucosal repair following intestinal damage will help to establish new approaches to CD therapy.

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Conflict of interest

None declared.

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Author contributions

All authors have made substantial contributions to the article. DO, JC, SC, CH and MDB: conception and design of the study, acquisition of data and/or analysis and interpretation of data. JH, RA, JVE and MDB: drafting the article or revising it critically for important intellectual content. All authors: final approval of the version to be submitted.

Supplementary data

Supplementary data to this article can be found online at ECCO-JCC online.

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