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MACROPHAGES FROM CROHN'S DISEASE PATIENTS EXHIBIT DEFICIENT REPAIR FUNCTIONS

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par

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pour Le Doyen
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Abstract

Background: Mucosal healing is becoming a major goal in the treatment of Crohn's disease. It has been previously reported that myeloid cells induce mucosal healing in a mouse model of acute colitis. The aim in this study is to investigate the pro-repair function of myeloid cells in healthy donors (HD) and Crohn's disease patients (CD).

Methods: Peripheral blood mononuclear cells (PBMC) from HD and CD patients were isolated from blood samples and tested either directly or after differentiation *ex-vivo* into macrophages (Mφ). Intestinal macrophages (IMACs) were isolated from the bowel mucosa of patients undergoing intestinal surgical resections. Through an *in vitro* wound healing assay the repairing ability of these various human myeloid cells and the mechanisms responsible of wound healing were evaluated.

Results: PBMC and myeloid $CD14^+$ cells from HD and CD were not able to repair at any tested cell concentration. Mφ from HD and ulcerative colitis (UC) patients were able to induce wound healing and this capacity was partially mediated by Hepatocyte Growth Factor (HGF). Remarkably, CD Mφ were unable to promote wound healing and produced lower levels of HGF as compared to Mφ from HD or UC patients. In particular, Mφ from CD in active phase (ACD) exhibited the weakest repair function, but this defect was rescued if rh-GM-CSF was added during the differentiation of PBMCs. Interestingly, IMACs from HD promoted wound healing and produced HGF.

Conclusion: We demonstrated that CD Mφ, unlike HD or UC Mφ, were defective in promoting wound healing, in particular if coming from an ACD. This deficient pro-repair function was related to a lower production of HGF. IMACs from HD colonic mucosa induced wound healing, confirming the results obtained with Mφ. Our results are in keeping with the current theory of CD as an innate immunodeficiency. In this context, Mφ may be responsible

for the mucosal repair defects observed in CD patients and for the subsequent chronic activation of the adaptive immune response.

Abbreviations

Ab: Antibody

A CD: Active Crohn's Disease

BSA: Bovine Serum Albumin

CD: Crohn's Disease

CFU: Colony Forming Unit

DAB: 3,3'-Diaminobenzidine

DSS: Dextran Sulphate Sodium

ECs: Epithelial cells

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme-Linked Immunosorbent Assay

FCS: Foetal Calf Serum

GM-CSF: Granulocyte Macrophage Colony Stimulating Factor

HD: Healthy Donor

HGF: Hepatocyte Growth Factor

HkEc: Heat killed E. coli

IMACs: Intestinal macrophages

Mφ: Macrophages

PBMCs: Peripheral Blood Mononuclear Cells

PBS: Phosphate Buffer Saline

PFA: Paraformaldehyde

P/S: Penicillin/Streptomycin

R CD: Remission Crohn's Disease

UC: Ulcerative Colits

Background

Crohn's disease (CD) is a chronically relapsing inflammatory condition of the gastrointestinal tract characterised by transmural segmental inflammation and mucosal ulcers. These features can lead to severe complications such as stenoses, fistulas, perforations and bleeding.[1] As a consequence 75-90% of CD patients will eventually need hospitalizations and/or surgical interventions. There remain numerous gaps in our knowledge of the immunopathogenetic events leading to intestinal inflammation and its consequences.

Several susceptibility genes have been identified for CD, as CARD15/NOD2, IBD5, IL23R and ATG16L1 and recent genome-wide association studies (GWAS) identified more than 60 gene polymorphisms that increase susceptibility for this disease (Lee and Parkes, Briefing in Functional Genomics, 2011), but they failed to contribute to the determination of the hereditable risk.

Many different factors seem to be involved in the CD pathogenesis. The genetic background, together with environmental factors including intestinal microflora, smoking habits, infections and dietary habits, can lead to abnormal innate and adaptive immune responses directed towards the intestinal flora and eventually leading to the destruction of the intestinal mucosa.[2]

Since there is no cure for CD, the goals of treatment are to induce remission, to maintain it, to minimize side effects, and to improve the quality of life. Classical medications for treating CD include corticosteroids, antibiotics or immune-modulators[1].

In the last few years, after the introduction of biological treatments, there has been increasing interest on the impact of mucosal healing (MH) in the outcome of the disease. In particular the endoscopic substudy of the ACCENT I clinical trial highlighted the relationship between mucosal healing and lower rates of patient hospitalisations[3]. New evidence showed that healing of the colonic mucosa was associated with prolonged remission rates, lower rates of complications, less need for steroids in the follow-up and fewer surgical interventions[4-6].

Collectively these clinical data suggest that promoting MH should become a new therapeutic goal in CD.

The murine Dextran Sulphate Sodium (DSS) colitis model is considered a very useful tool to investigate the mechanisms involved in wound healing and characterize the cell populations involved in this process because DSS has a direct toxic effect on intestinal epithelial cells, causing erosions and ulcers followed by acute colonic inflammation.[7]

In our previous work we provided evidence that GM-CSF (Granulocyte-Macrophage Colony-Stimulating Factor) promoted mucosal repair, dampened colon inflammation and ameliorated the clinical signs of colitis in the DSS colitis model. Remarkably, we observed that GM-CSF-expanded monocytic $CD11b^+$ cells promoted both in vivo and in vitro wound re-epithelialisation, suggesting that myeloid cells are instrumental in MH[8]. We further demonstrated that macrophage-depleted or GM-CSF-receptor-KO (GM-CSFRKO) mice were deficient in ulcer healing (manuscript in progress).

Some authors have hypothesized a role of the innate immune system in the pathogenesis of CD, proposing a model of innate immune deficiency[9]. It was demonstrated that CD patients have an impaired acute inflammation leading to a defective neutrophil recruitment. This was proved in two different models, first evaluating the acute inflammatory response following acute trauma in the colonic mucosa[10] and then after subcutaneous injection of killed *Escherichia coli* in healthy controls or CD patients[11]. This impaired neutrophil accumulation may lead to an incomplete removal of bacteria and consequently to increased phagocytosis by macrophages (Mφ) with formation of granulomas[9, 10, 12]. Moreover, Mφ derived from CD patients and challenged with heat-killed *E.coli* (HkEc) show diminished production of pro-inflammatory cytokines[11].

Following these data, some authors proposed to stimulate the intestinal innate immunity in CD with GM-CSF, a cytokine known to promote myeloid cells growth, differentiation and

function, and already known to ameliorate the disease in mice models [8, 13]. GM-CSF was investigated first in an open-label dose-escalation trial on 15 patients with moderate to severe CD (CD Activity Index, CDAI, greater than 200 and lower than 475) and the treatment resulted in a significant decrease in mean CDAI after 8 weeks of treatment with negligible side effects[14]. A larger randomized, placebo-controlled trial was then performed including 124 patients with CDAI between 200 and 475 and treatment with GM-CSF resulted to an increased remission rate, lower median CDAI, improved quality of life and improved mucosal healing[15].

In the light of these insights, we decided to investigate further the features of myeloid cells coming from CD patients, in particular regarding their role in mucosal healing (MH).

Materials and Methods

PBMCs isolation and Macrophages differentiation, culture and stimulation

Peripheral venous blood was collected from patients or healthy donors (HD) in tubes containing EDTA. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation and then $CD14^+$ cells were positively selected by magnetic sorting (Anti-human magnetic particles-DM- Clone MφP9- BD Biosciences). Cells were plated at density of $0.5\text{-}1x10^6$ cells/well in 12-well plates suspended in RPMI complete medium (L-Glutamine, 25mM HEPES, 10% FCS, 1% P/S). At day one and 4, non-adherent cells were discarded. At day 5, adherent macrophages (Mφ) were recovered by scraping.

Where indicated, at day 5, Mφ were activated for 24 h with HkEc with a ratio 1:50 Mφ/CFU.

Where indicated, HD and CD PBMCs $CD14^+$ were cultured for 5 days at 37°C and 5% CO_2 in the presence of 10 ng/ml recombinant-human GM-CSF (rh-GM-CSF) (R&D) or goat anti-human GM-CSF antibody (R&D) at the concentration of 2 μg/ml.

In vitro wound healing assay (Fig. 1)

To test the repairing ability of myeloid cells, an *in vitro* wound healing model was used. Human intestinal carcinoma Caco.2 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS and 1% Penicillin/Streptomycin to confluent monolayers in 12 well plates at 37°C and 5% CO_2. Caco.2 monolayers were starved for 24 h in 0.5% FCS DMEM and then wounded with a p1000 plastic pipette tip connected to a vacuum aspirator, to produce reproducible circular wounds with an average size of 0.8-1.4 mm^2 [16]. Epithelial cells were then incubated for 3 more days to observe wound healing. As negative control we used serum deprived medium (DMEM 0.5% FCS), whereas DMEM 10% FCS was our positive control (Fig.1)

On day 3, epithelial cell monolayers were fixed for 10 minutes with 4% paraformaldehyde (PFA) and stained for 4 min with Groat Haematoxylin.

Pictures were taken using a 4x objective (microscope Olympus IX81) at day 0 (day of wounding) and at day 3 and wounded areas measured using Photoshop software[17]. Wound healing was assessed as the percentage of re-reepithelialised area at day 3 as compared to day 0.

To evaluate the wound healing ability of myeloid cells, PBMCs, $CD14^+$ or $CD14^-$ cells or Mφ isolated from CD patients or HD were added at day 0 in different Mφ: Caco.2 ratios (1:5, 1:10, 1:100) on top of wounded epithelial monolayers[18].

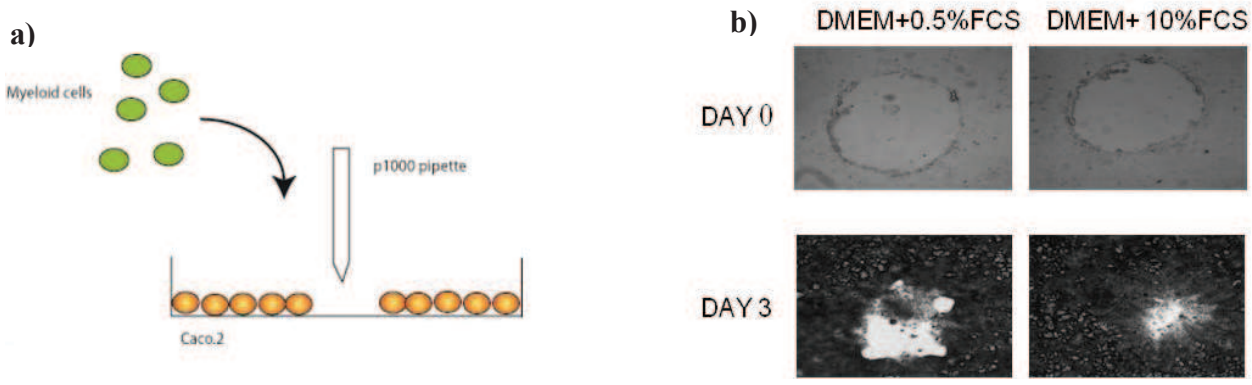


Fig. 1- *In vitro* wounding assay

a) Our *in vitro* model
b) Representative pictures of wounded monolayers taken at day 0 (upper row) and at day 3 (lower row). The left column represents the negative control; the right column is the positive control

Flow cytometric analysis of Mφ

Mφ were surface-stained with monoclonal antibodies to CD14 (clone M5E2, BD Biosciences), CD11b (clone ICRF44, BD Biosciences), CD11c (clone B-ly6, BD Biosciences), CD16 (clone 3G8, BD Biosciences), HLA-DR (clone G46-6, BD Biosciences), CD33 (clone WM53, BD Biosciences), CX_3CR1 (clone 2A9-1, MBL), CD116 (clone

hGMCSFR-M1, BD Biosciences), CD68 (clone Y1/82A, BD Biosciences). Data were acquired using a FACSscan flow cytometer (BD Biosciences) and analysed with CellQuest software (BD Biosciences). Dead cells were excluded by Propidium Iodide staining.

F-actin formation

To quantify F-actin formation in epithelial cells (ECs), Caco.2 cells, fixed as described above, were permeabilized for 10 minutes with 0.3% Triton X-100/PBS, incubated in a blocking solution (2% bovine serum albumin/PBS) for 30 minutes at room temperature (RT) and then incubated with Alexa Fluor 488 Phalloidin (Invitrogen A12379) for 20 minutes. After staining, the cells were washed in PBS and analyzed with a fluorescence microscope (Olympus IX81) where digital pictures were taken. The density of Phalloidin staining was quantified by Photoshop software.

Cell proliferation assay

PFA-fixed cells were permeabilized for 10 minutes with 0.2% saponin/PBS, incubated at RT in a blocking solution (5% normal goat serum/PBS) for 60 minutes and then incubated with mouse-α-human Ki67 (nuclear Ab against the nuclear protein KI-67, clone MM1, Novocastra) for 1 hour, followed by washing in PBS and staining with a secondary antibody goat-α-mouse Alexa Fluor 488 (Mol. Probes) for 30 minutes. After staining, cells were washed in PBS, counterstained with Dapi for 5 minutes and then analyzed with a fluorescence microscope where digital pictures were taken. The number of Ki-67 positive cells present around the wound edge was quantified by Photoshop software.

HGF Enzyme-Linked Immunosorbent Assay (ELISA)

HGF concentration was determined by Enzyme-Linked Immunosorbent Assay (ELISA) using the Duo Set ELISA development system by R&D Systems. Briefly, 100 µl of undiluted supernatant was added to wells pre-coated with a mouse anti-human HGF Ab and blocked with 1% BSA in PBS. After incubation and washing, the wells were incubated with a biotinylated goat anti-human HGF Ab, re-washed and then incubated with streptavidin conjugated to horseradish-peroxidase. Finally the substrate solution and then the stop solution were added and the optical density was measured at 450 nm. Each sample was tested in duplicates.

Immunohistochemistry

Endoscopic biopsies were recovered, fixed in 10% buffered formalin and embedded in paraffin. Samples were then cut in serial sections of 4µm of thickness and mounted on slides. Slides were hydrated, washed and quenched in 3% H_2O_2/PBS to remove endogenous peroxidase. Slides were then washed and incubated in an antigen retrieval solution (sodium citrate at pH=6). The primary antibodies were added (rabbit anti-human HGFα, Santa Cruz and mouse anti-human CD68, clone PGM-1, Dako) and incubated overnight at 4°C. After washing, the secondary Ab (rabbit envision HRP, DAKO) was incubated for 30 minutes at RT and after further washing, 3,3'-Diaminobenzidine (DAB kit from Vector) was added to reveal HGF. Revelation was stopped by washing with tap water.

Slides were then washed in PBS and incubated for 30 minutes with the secondary antibody for CD68, a goat anti-mouse biotin (Amersham, RPN 1177). After washing, slides were incubated for 30 minutes in Streptavidin Alkaline Phosphatase (AP) and, after further washing, CD68 was revealed by incubation for 15 minutes with 5-Bromo-4-chloro-3-indolyl

phosphate/ nitro blue tetrazolium (BCIP/NBT, Roche). The reaction was stopped with NTMT solution (NaCl 5M- Tris 2M- $MgCl_2$2M- Tween 20 and distilled H_2O).

Slides were then washed, dehydrated and embedded. Pictures were taken with a microscope Eclipse 50i connected to a camera DS-Fi 1.

This study has been approved by the human research ethics committee (protocol 41/11).

Real-time Quantitative Reverse-transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated using RNeasy Plus Micro kit (Qiagen, Valencia, CA). Sample quality was tested on agarose gels and absence of genomic DNA assessed by PCR using primers specific for the housekeeping gene ALG-9 (unknown sequence, Qiagen). Total RNA samples were then submitted to reverse transcription using the ThermoScript RT-PCR system (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol, and oligo-dT as primers. PCR amplification was performed on a MyiQ iCycler (Bio-Rad, Hercules, CA) using the iQ SYBR Green Supermix (Bio-Rad). Primers specific for HGF (sense: 5´AAAGGACTTCCATTCACTTGC; antisense:5´CGCTCTCCCTTACTCAAGCTA) were purchased from Microsynth (Balgach, Switzerland). Primer pairs designed for QuantiTect Primer assays (unknown sequence, Qiagen) were used for quantification of c-Met. For each individual sample, mRNA quantification was performed by normalizing the number of mRNA copies obtained for the gene of interest per million of mRNA copies obtained for ALG-9.

Isolation of intestinal macrophages (IMACs)

Surgical intestinal specimens were collected and washed with PBS; the mucosa was released from the muscular layer and then stirred for 30 minutes in HBSS+DTT to free it from mucus. The mucosa was then stirred in Hank's Buffered Salt Solution (HBSS) + EDTA 0.1M at 200

rpm at 37°C for 30 minutes. After rinsing, epithelial cells were detached by vortexing and vigorous shaking. The mucosa was transferred in DMEM + 10% FCS and kept incubated overnight at 4°C. The following day, after washing with PBS, mucosal slices were digested in PBS + Ca^{2+} and Mg^{2+} with collagenase (Sigma), hyaluronidase (Sigma) and DNase (Roche) and stirred for 60 min at 37°C at 300 rpm. The cell suspension obtained was then poured through a strainer and slices were discarded following further vortexing and vigorous shaking to extract the highest possible number of cells. Intestinal monocitic cells were isolated by Ficoll density gradient centrifugation and $CD33^{+}$ cells were positively selected by magnetic sorting (mouse anti-human magnetic micro beads, clone AC104.3E3- Miltenyi Biotec)[19].

This study has been approved by the human research ethics committee (protocol 41/11).

Statistical analysis

Statistical analyses to compare two groups were performed using a two-tailed Mann-Whitney test, which doesn't assume a Gaussian distribution (Graph Pad Prism Software). Comparison between several groups to identify a trend was performed by a One-way ANOVA test with Trend test as post test. Limit of significance was considered for $p<0.05$.

Patients

All patients' baseline characteristics are listed in table 1.

CROHN'S DISEASE				
Patient	Age	Sex	Status of disease	Therapy
1	29	M	Remission	MTX
2	49	M	Remission	IFX
3	28	F	Remission	Steroids
4	58	M	Remission	NO TP
5	29	M	Remission	IFX
6	22	M	Remission	IFX
7	41	F	Remission	UN
8	35	M	Remission	IFX
9	14	M	Remission	IFX
10	62	M	Remission	IFX
11	31	F	Remission	IFX
12	40	M	Remission	IFX
13	40	F	Remission	IFX
14	46	M	Remission	IFX
15	45	F	Active	IFX
16	25	F	Active	IFX
17	24	F	Active	IFX
18	45	F	Active	IFX
19	56	M	Active	Protocol Neovacs
20	56	M	Active	Protocol Neovacs
21	29	M	Active	UN
22	46	F	Active	Steroids + Azathioprine
23	46	M	Active	IFX
24	24	M	Active	IFX
25	35	M	Active	UN
26	24	F	Active	IFX
27	48	F	Active	IFX+ Antibiotics
28	64	M	Active	Steroids + MTX
ULCERATIVE COLITIS				
29	54	M	Remission	Mesalasine
30	84	M	Remission	Mesalazine
31	43	F	Remission	Mesalazine
32	23	F	Remission	mercaptopurine
33	62	F	Remission	NO TP
34	31	F	Remission	IFX
35	43	M	Remission	IFX
36	48	M	Remission	NO TP
37	32	F	Remission	IFX
38	44	M	Remission	IFX
39	49	M	Remission	NO TP
40	21	F	Remission	IFX
41	47	F	Remission	Mesalazine
42	26	F	Active	IFX
43	61	M	Active	IFX
44	44	M	Active	IFX
45	34	M	Active	UN
46	71	F	Active	NO TP

IFX= infliximab
MTX= Methotrexate
UN= Unknown

Table 1- Patients' baseline characteristics.

Results

1. Role of monocytic cells in wound healing

1.1 Role of PBMC, $CD14^+$ or $CD14^-$ cells

Caco.2 monolayers were wounded and total HD PBMCs, $CD14^+$ (marker of monocytes) or $CD14^-$ cells added on top of wounded epithelial cell monolayers at increasing concentrations ($5x10^3$, 10^4, $2.5x10^4$, 10^5, $5x10^5$). We found no amelioration in the percentage of repair for any concentration of PBMCs, $CD14^+$ or $CD14^-$ cells added, as compared to controls (Fig.2).

CD total PBMCs as well as magnetically sorted CD $CD14^+$ or $CD14^-$ cells, showed no difference with HD total PBMCs, $CD14^+$ or $CD14^-$ cells (data not shown) and we concluded that neither HD nor CD PBMCs could improve wound healing.

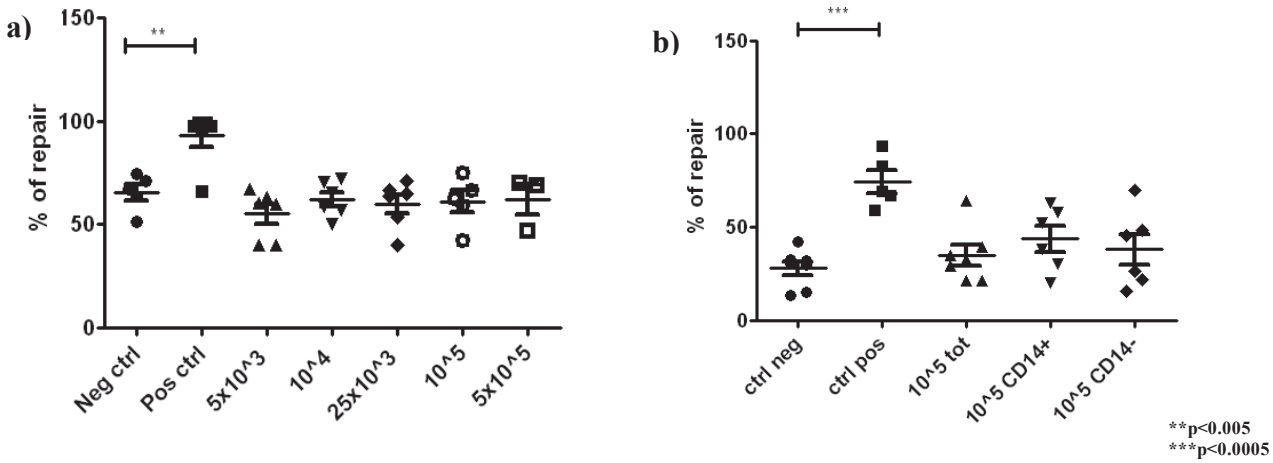


Fig.2- No effect of HD PBMCs on epithelial cell repair with (n=6).
Each dot represents a single HD tested in separate experiments
a) HD PBMCs were added at increasing concentration
b) HD PBMCs were added after magnetic sorting for $CD14^+$or $CD14^-$

1.2 Role of macrophages in wound healing

1.2.1 HD macrophages

Based on previous results showing defective functionality of CD Mφ in pro-inflammatory cytokine production, likely leading to an impaired neutrophil recruitment [10, 11], we sought to investigate further the role of these cells in our wounding assay. HD $CD14^+$ cells were differentiated *ex-vivo* into Mφ as described in Materials and Methods and then added at increasing concentrations ($5x10^3$, 10^4, $5x10^4$, 10^5) on wounded Caco.2 cells. We observed an amelioration of repair if Mφ were added compared to the control and the percentage of healing was increased with the number of Mφ (Fig. 3 a-b).

To extend further our data, HD Mφ were activated with heat- killed *E. coli* (HkEc) for 24 hrs (ratio Mφ:CFU=1:50) before being added to our assay. Active Mφ have specifically increased functional activity, like killing of intracellular parasites, tumour cells lysis and maximal secretion of inflammatory mediators[20].

To confirm the activation status of the Mφ, a quantitative PCR was performed to investigate the mRNA expression level of Interleukin-6 (IL-6) after stimulating the Mφ with HkEc, LPS (1μg/ml) or CpG oligodeoxynucleotides (CpG ODN, 5μM). We tested 4 HD for each condition and there was a significant increase in IL-6 encoding mRNA copy numbers in HkEc (mean=371±28.37) and LPS (mean=138±9.6) activated Mφ as compared to not activated (NA) Mφ (mean= 21.65±8.9, $p<0.005$ and $p<0.05$, respectively), but there was no increase in CpG ODN activated Mφ (mean= 22.9±2.8, $p<0.9$).

We then tested in our assay the same patients and the same Mφ concentrations as in Fig.3 in 5 separate experiments. Once activated with HkEc, HD Mφ were indeed able to induce wound repair even at the lowest concentration of $5x10^3$ compared to our negative control ($p<0.005$).

We decided also to test if other stimuli than HkEc were able to induce the same pro-repair activity. $5x10^4$ HD Mφ were incubated with either HkEc as before or LPS (1μg/ml) or CpG ODN (5μM) and then their pro-repair function was evaluated in the wounding assay. HkEc resulted to be the only effective activator; LPS induced a small amelioration of repair but without reaching a statistical significance; CpG ODN had no effect (Fig.3 c).

From these experiments we concluded that human Mφ are indeed able to promote wound healing in our in-vitro model and these cells can better perform if activated with a very strong stimulus, like HkEc.

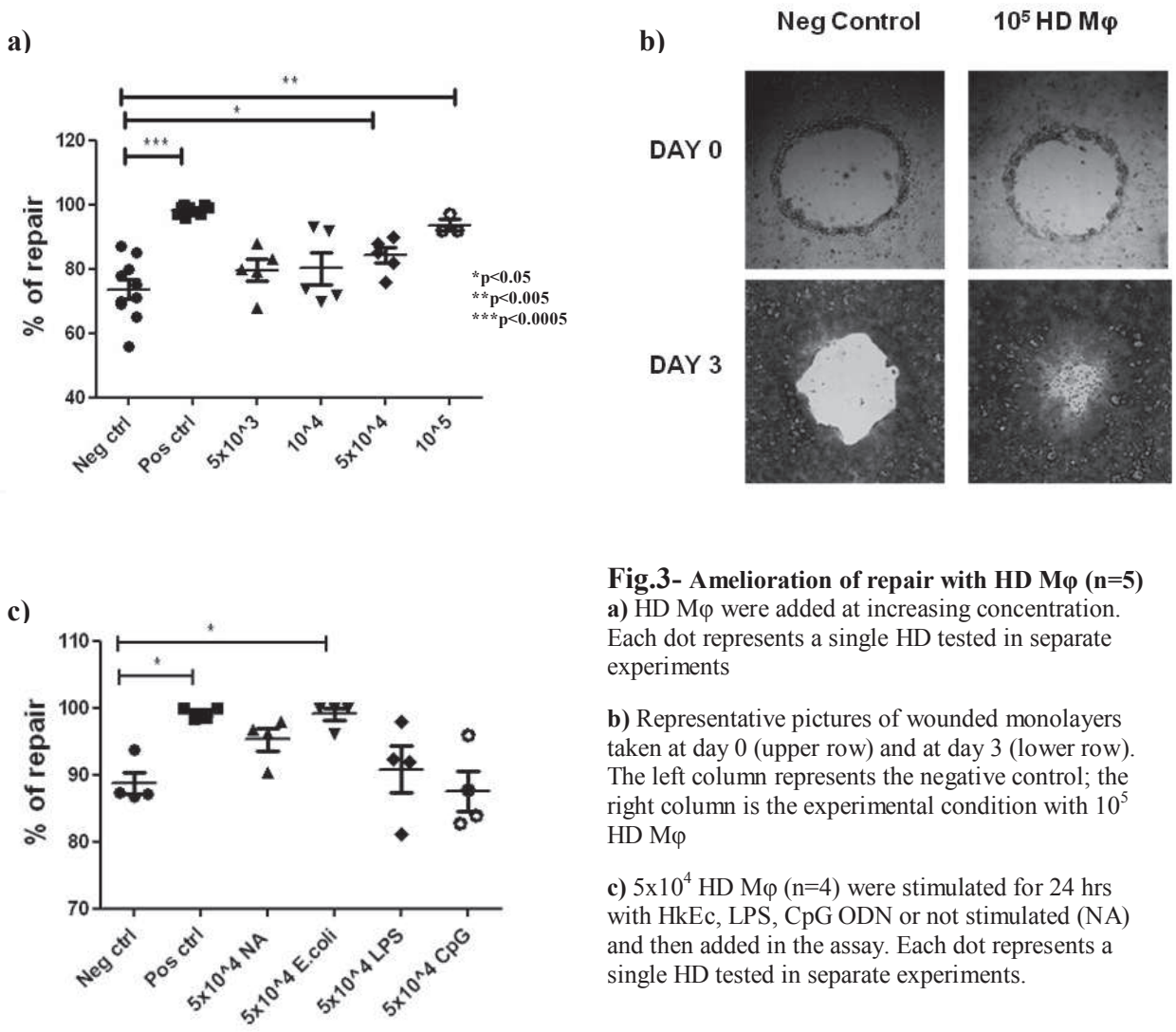


Fig.3- Amelioration of repair with HD Mφ (n=5)
a) HD Mφ were added at increasing concentration. Each dot represents a single HD tested in separate experiments

b) Representative pictures of wounded monolayers taken at day 0 (upper row) and at day 3 (lower row). The left column represents the negative control; the right column is the experimental condition with 10^5 HD Mφ

c) $5x10^4$ HD Mφ (n=4) were stimulated for 24 hrs with HkEc, LPS, CpG ODN or not stimulated (NA) and then added in the assay. Each dot represents a single HD tested in separate experiments.

1.2.2 CD macrophages

We then investigated if Mφ isolated from CD patients exhibited the same pro-repair function as HD Mφ. CD $CD14^+$ cells were isolated from PBMC, differentiated *ex-vivo* into Mφ and then added to our wounding assay at increasing concentrations ($5x10^3$, 10^4, $5x10^4$, 10^5), as previously described. This time we could observe no amelioration of wound healing at any concentration tested. In fact, there was a significant difference in the percentage of re-reepithelialised surface between 10^5 HD Mφ and 10^5 CD Mφ (Fig. 4). However, when CD Mφ were activated with HkEc, their pro-repair function was restored.

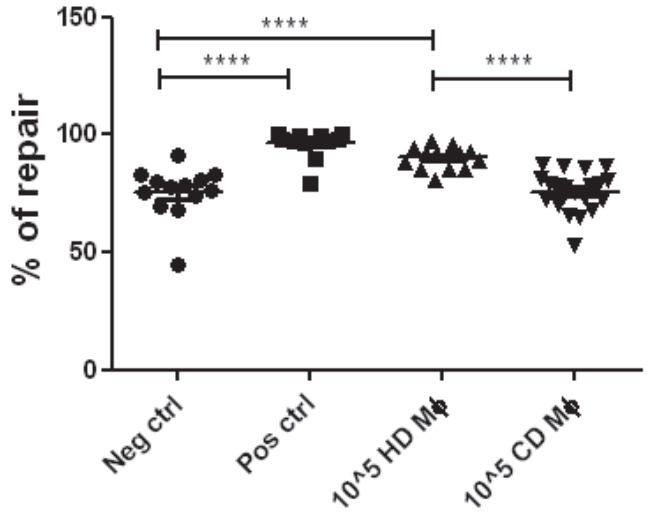


Fig.4- CD (n=22) Mφ exhibit a deficient pro-repair function as compared to HD (n=14).
Each dot represents a single individual tested in a separate experiment

****p<0.0001

We then evaluated if there was any relationship between the pro-repair function of CD Mφ and the status of the disease. Indeed, we found that Mφ isolated from CD patients in active phase had a decreased repair function than those isolated from patients in remission, even though neither of these Mφ populations could repair significantly better than the negative control (Fig.5).

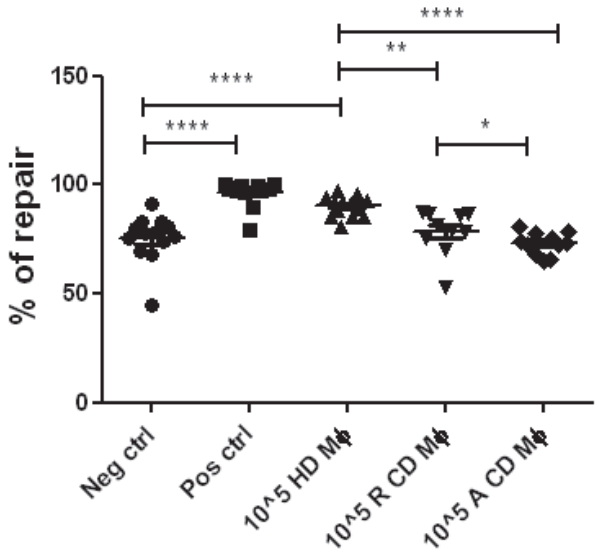


Fig.5- Mφ from CD patients in acute phase (A CD, n=11) exhibit a worse repair function than Mφ of CD in remission (R CD, n=11).
Each dot represents a single individual tested in a separate experiment

***p<0.05**
****p<0.005**
*****p<0.0005**
******p<0.0001**

We wondered if this defective pro-repair function found in CD Mφ was applicable also to Mφ isolated from UC patients. Interestingly, UC Mφ were able to promote wound healing in our assay significantly better than our negative control and CD Mφ (Fig. 6 a-b). As we detected a lower repairing ability for ACD as compared to RCD Mφ, we investigated if this defect was also applicable to UC Mφ. A UC Mφ repaired as well as R UC Mφ and there was no difference with R UC or HD Mφ (Fig. 6 c).

Overall we conclude that CD Mφ exhibit a deficient pro-repair function as compared to HD Mφ and this deficiency is more evident if the patient is in the active phase of the disease. On the other hand, UC Mφ do not seem to share this lack of pro-repair activity, neither when obtained from patients in remission nor when obtained from patients with active disease (Fig. 6 c).

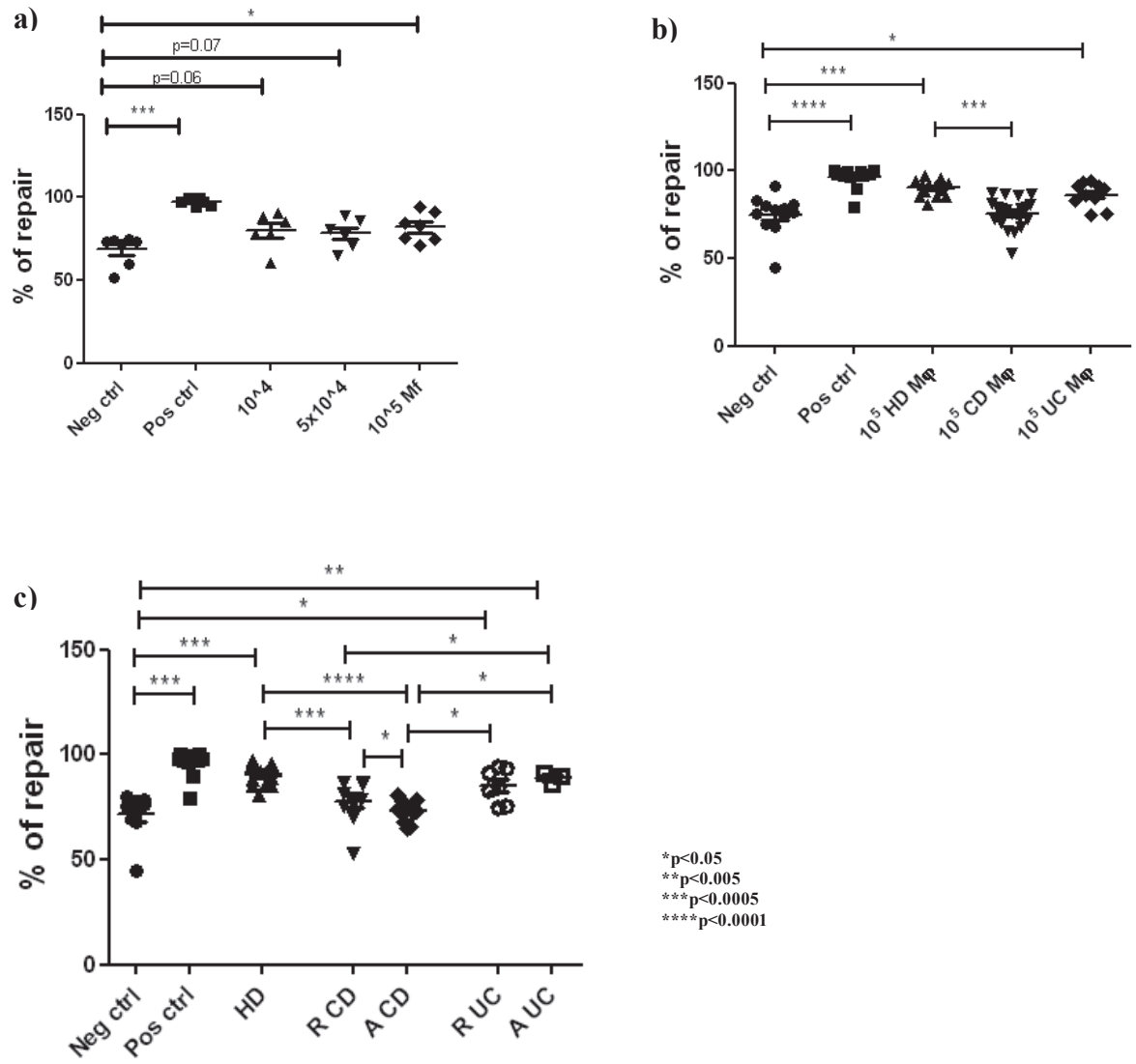


Fig.6- UC Mφ are able to induce wound healing
Each dot represents a single individual tested in a separate experiment

a) UC Mφ (n=7) tested at increasing concentrations

b) UC Mφ (n=10) repair better than CD Mφ (n=22), and as well as HD Mφ (n=14)

c) Comparison of all groups tested: HD (n=14), RCD (n=10), ACD (n=11), RUC (n=7), AUC (n=3)

2. Phenotypical characterization of HD and CD Mφ

Having demonstrated a functional difference between HD and CD Mφ, we wondered if this was associated with a phenotypical diversity of the two populations. To investigate the markers expressed by these cells, we stained them with a panel of myeloid markers (CD14, CD11b, CD11c, CD16, HLA-DR, CD33, and CD68), the fractalkine receptor CX_3CR1, and the GM-CSF receptor CD116 and analysed them by flow cytometry. We tested a total of 9 CD patients and 6 HDs in 6 separate experiments. CD14 (HD 24%, CD 42%), CD11b (HD 35%, CD 42%) and HLA-DR (HD 46%, CD 47%) were the highest expressed markers while CD16 was the lowest expressed (HD 1%, CD 4%). CD33, CX_3CR1 and CD116 were expressed at lower percentages by CD Mφ compared to HD (6%, 6% and 13% respectively, versus 16%, 15%, 24%), but these differences didn't reach statistical significance, probably in part because of the variability amongst HDs and CDs samples (Fig.7).

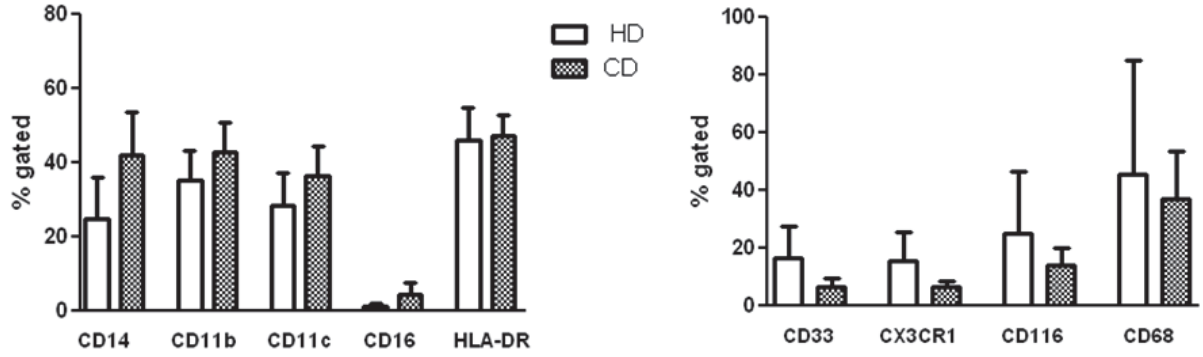


Fig.7- Phenotypical characterization of macrophages
Mφ from HD (n= 6) and CD (n=9) were compared by flow cytometry. No statistically significant difference was found

3. Characterization of macrophage-induced epithelial repair *in vitro*

3.1 Is cell contact between epithelial cells (ECs) and Mφ necessary to promote wound healing ?

To characterize further the mechanisms through which Mφ exert their healing function, in particular to determine if there was need for a cell-cell contact between epithelial cells (ECs) and Mφ to trigger repair, we introduced transwells in our assay. In the basolateral chamber Caco.2 cells were cultivated and wounded as previously described, while 10^5 HD Mφ, shown in our previous experiments to induce repair most effectively, were added in the apical chamber. Interestingly, an amelioration of the repair was observed, as compared to the control (Fig 8). We could therefore conclude that cell contact is not necessary in our model to induce wound healing, suggesting that soluble factors are involved in this process.

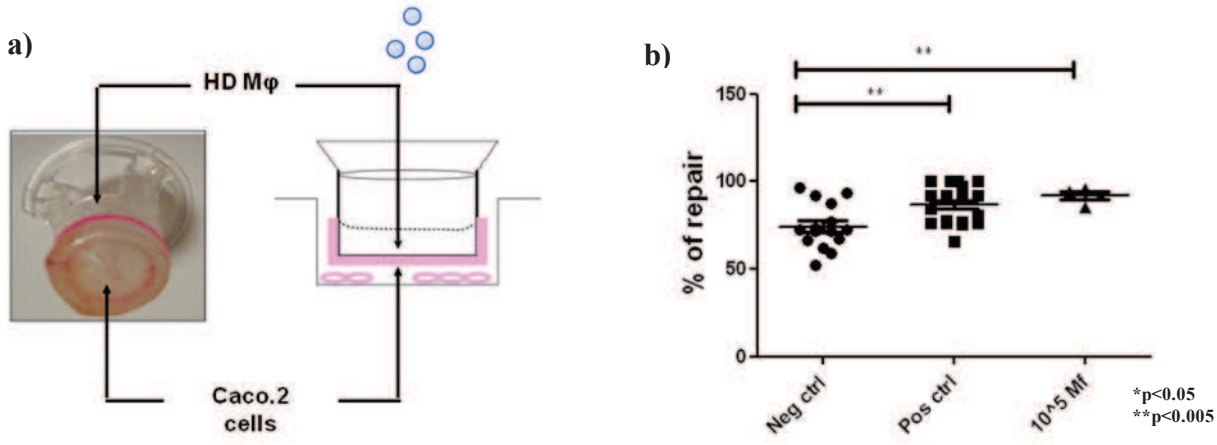


Fig.8 - HD Mφ are able to induce wound healing without contact with ECs
a) Model of transwell: HD Mφ are added in the upper chamber, while Caco.2 cells lie in the lower chamber
b) HD Mφ (n=4) induce repair without contact with Caco.2 cells
Each dot represents a single individual tested in a separate experiment

3.2 Role of restitution and proliferation in our model

An efficient wound repair in the bowel mucosa is essential to insure mucosal integrity in the gut. After an injury, the epithelial cells surrounding the wound begin first to migrate towards the centre of the denuded area within minutes; this process is called restitution and doesn't involve proliferation. Then cells start to proliferate to replenish the cell pool and eventually they mature into differentiated epithelial cells[21].

In the light of these elements, we investigated into whether the wound healing promoted by human Mφ observed in our assay was either due to a restitution process, to proliferation or to both.

3.2.1 Role of Restitution

To evaluate restitution, we quantified the formation of the F-actin purse string around the wound edge. Indeed, after injury, the epithelial sheet responds by reorganizing his actin cytoskeleton into a belt around the wound edge, called purse string. This string pulls the surrounding cells towards the centre of the lesion flattening these cells with cytoplasm protrusions (lamellae) that extend to cover the denuded area[17, 22]. To test restitution, Caco.2 cells were cultivated and wounded as previously described and then 10^5 HD Mφ were added in transwells. At day 3, ECs were stained with Alexa Fluor 488 Phalloidin as described in Materials and Methods and the intensity of the phalloidin staining in the purse string was quantified. We observed an increase in F-actin expression around the wound edge in the wells where Mφ were added, as compared to the negative control and could clearly visualize the lamellae protruding towards the centre of the denuded area, lamellae which were absent in the negative control wells. We could therefore conclude that restitution takes place in our wound healing model (Fig.9 and Fig.10).

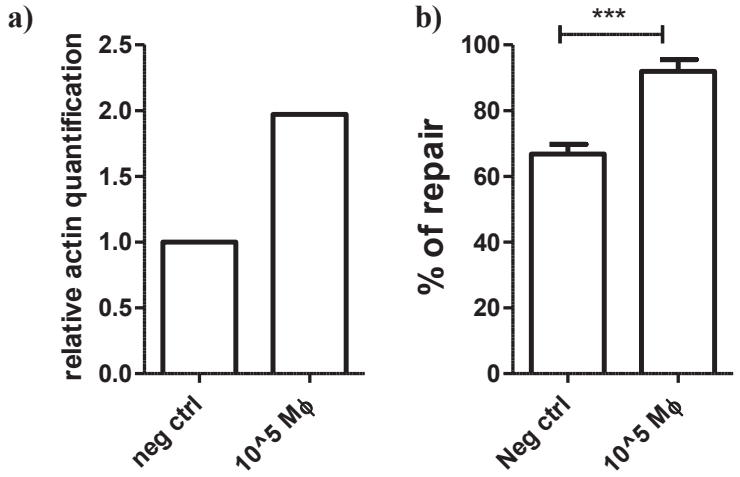


Fig.9- Relationship between F-actin expression and wound healing.

One example out of 3 separate experiments is represented
a) Quantification of F-actin expression in the purse string.
b) Percentage of restitution in the same experimental setting

***p<0.0005

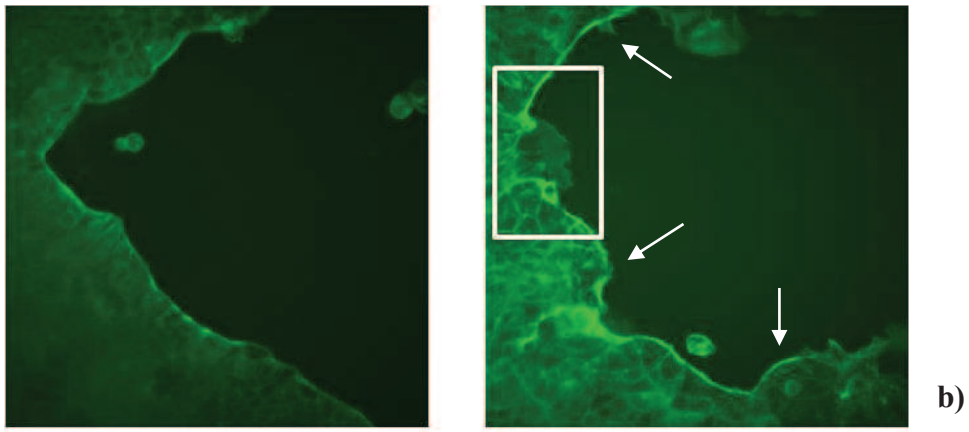
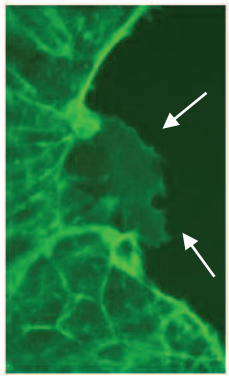


Fig.10- F-actin expression with immunofluorescent staining

a) Negative control (DMEM 0.5%FCS)
b) Wounded Caco.2+ 10^5 Mφ. The purse string and the actin cytoskeleton are visible (white arrows)
c) Detail of b) to highlight the formation of the lamellae (white arrows)



3.2.2 Role of proliferation

We thought next to determine if proliferation was involved in our experimental setting and if it would be induced by HD Mφ.

Caco.2 cells were cultivated and wounded as previously described and 10^5 HD Mφ were added in transwells. At day 3 ECs were stained for Ki-67 (Ab against the nuclear protein Ki-67, expressed in proliferating cells) as described in Materials and Methods, and the numbers of Ki-67^+ cells at the wound border were counted. A total of 5 different HD were tested and the results expressed as a relative ratio of the total number of Ki-67^+ cells present in the negative control. There was no significant difference in the number of Ki-67^+ cells between the negative control and the wells with 10^5 HD Mφ (mean ratio 1 and 0.8, respectively, p=0.5) (Fig 11).

Overall, we could conclude that, in our model, wound healing mediated by HD Mφ is a restitution process with a visible actin purse string and without contribution of proliferation.

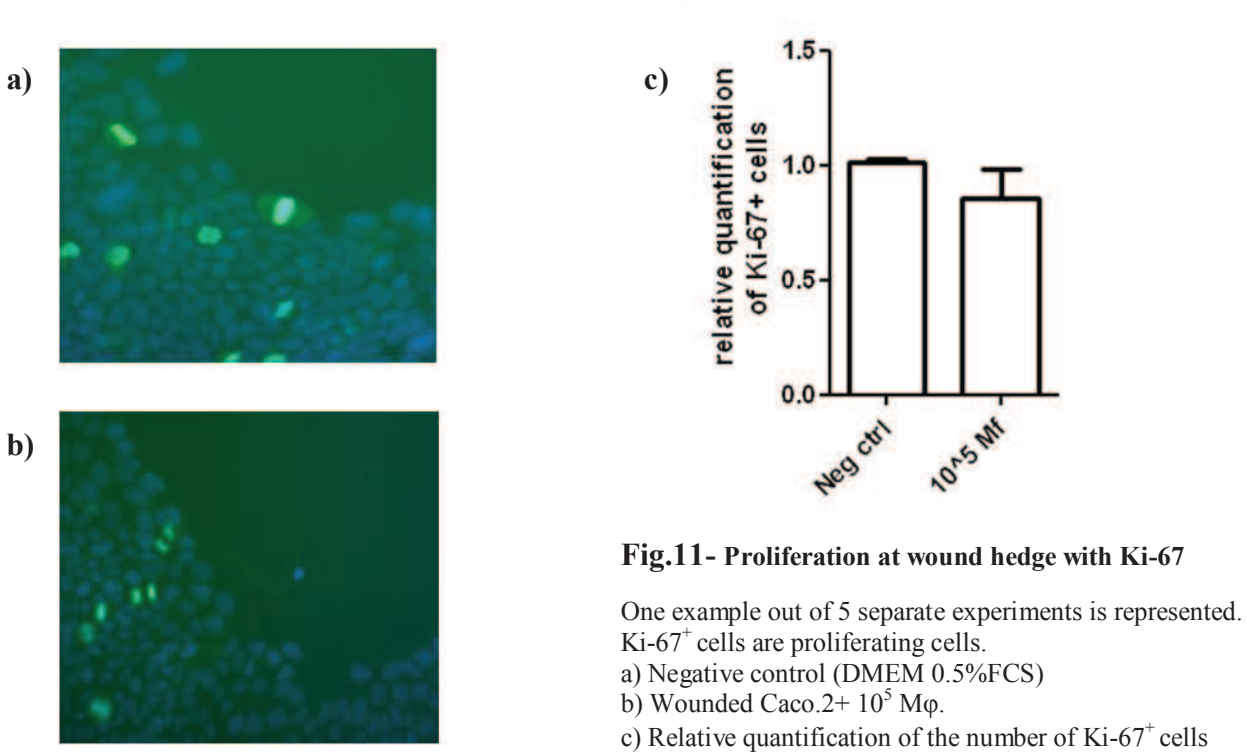


Fig.11- Proliferation at wound hedge with Ki-67

One example out of 5 separate experiments is represented.
Ki-67^+ cells are proliferating cells.
a) Negative control (DMEM 0.5%FCS)
b) Wounded Caco.2+ 10^5 Mφ.
c) Relative quantification of the number of Ki-67^+ cells

4. Role of Granulocyte Macrophage Colony Stimulating Factor (GM-CSF)

4.1 Role of GM-CSF on HD Mφ

In our previous work we provided evidence that GM-CSF therapy promotes mucosal repair, dampens colon inflammation and ameliorates the clinical signs of DSS mouse colitis. Remarkably, we provided evidence that GM-CSF-expanded monocytic $CD11b^+$ cells can promote both *in vivo* and *in vitro* epithelial repair[8]. The therapeutic role of GM-CSF has been also demonstrated in CD patients and it has been justified on the hypothesis of CD as an innate immunodeficiency[14, 15].

Therefore we decided to investigate into whether GM-CSF plays a role in macrophage-induced promotion of wound healing.

As we have previously demonstrated that HD PBMCs $CD14^+$ are not able to induce wound healing, we wondered if rh-GM-CSF could promote the repair if added directly in the co-cultures of wounded ECs + HD PBMCs at day of wounding. 10^4 and 10^5 PBMCs $CD14^+$ from 2 HDs were added in duplicates to the Caco.2 with or without rh-GM-CSF or anti-GM-CSF Ab. In our model there was neither amelioration nor inhibition of repair in these conditions. For 10^4 PBMCs $CD14^+$ the mean percentage of repair was 75% with PBMCs alone, 76% if rh-GM-CSF was added and 73% with anti-GM-CSF Ab, with a $p=0.54$, $p=0.39$ and $p=0.49$ respectively in comparison with the negative control. For 10^5 PBMCs $CD14^+$ the mean percentage of repair was 72% with PBMCs alone, 74% if rh-GM-CSF was added and 82% with anti-GM-CSF Ab, with a $p=0.40$, $p=0.57$ and $p=0.22$, respectively in comparison with the negative control. In the same set of experiments we also added directly rh-GM-CSF or anti-GM-CSF in the culture medium of wounded ECs, as controls to test if GM-CSF has a direct effect on Caco.2 cells. We could find neither an amelioration of repair nor a delay in healing ($p=0.288$ and $p=0.614$, respectively in comparison with the negative control).

Considering that GM-CSF is a growth factor influencing the growth, differentiation and survival of myeloid cells, we tested whether the incubation of HD $CD14^+$ cells with either rh-GM-CSF or anti-GM-CSF during their differentiation into Mφ, could influence the Mφ ability to promote healing. We found that neither rh-GM-CSF (Fig. 12 a) nor anti-GM-CSF (Fig. 12 b) had an effect on HD Mφ regarding their ability to induce wound repair.

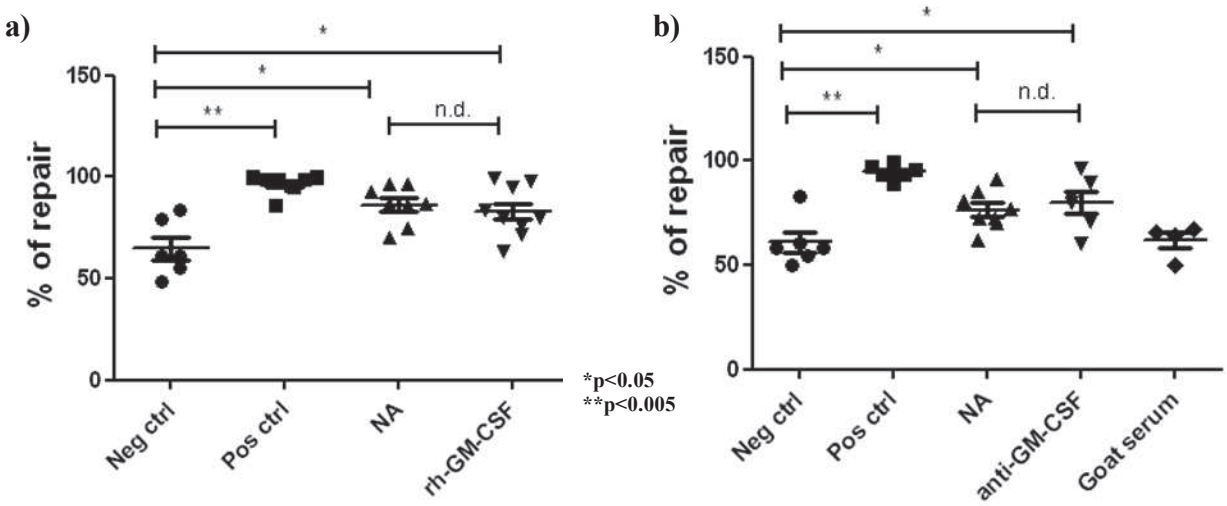


Fig.12- Ability of HD Mφ differentiated in presence of rh-GM-CSF (a) or anti-GM-CSF (b) to induce wound healing.
a) No difference in repairing ability is found for 10^5 HD Mφ (n=8) differentiated or not (NA) with rh-GM-CSF
b) No difference in repairing ability is found for 10^5 HD Mφ (n=7) differentiated or not (NA) with anti-GM-CSF. Goat serum was used as a control.
Each dot represents a single individual tested in a separate experiment.
The same set of experiment was performed also for $4x10^4$ and 10^4 Mφ, and produced similar results.

4.2 Role of GM-CSF on CD Mφ

CD Mφ were tested in our wounding assay after differentiation with rh-GM-CSF. We couldn't find any significant difference in the percentage of restitution mediated by Mφ if the total number of CD patients (n=8) was considered (Fig 13 a). However, interestingly, when we analysed separately patients in active phase and patients in remission, we found an amelioration of repair only if Mφ coming from patients in the active phase and differentiated

with rh-GM-CSF, were added in our assay. No amelioration of repair was observed if the same number of Mφ coming from patients in remission, even if differentiated with rh-GM-CSF, was added in our model (Fig.13 b).

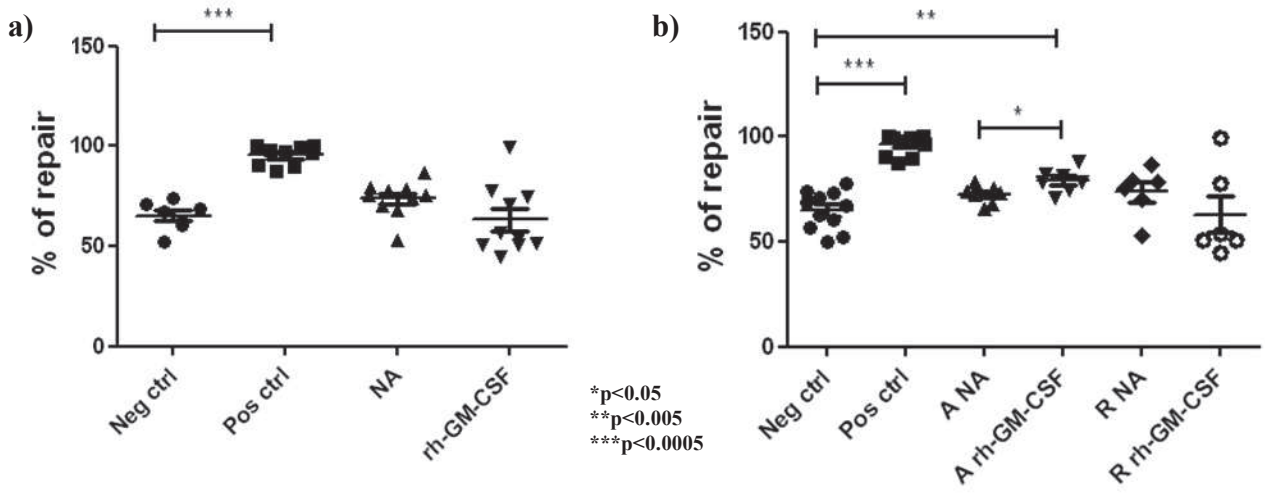
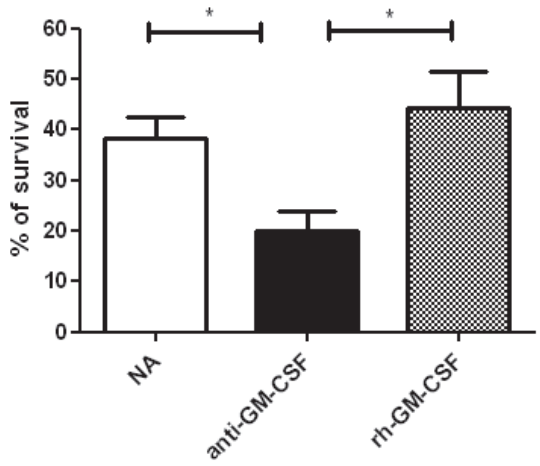


Fig.13- Addition of GM-CSF during differentiation of Mφ coming from patients in active phase ameliorates their ability to promote wound healing
a) Percentage of wound repair with 10^5 Mφ from CD patients (n=8) differentiated (rh-GM-CSF) or not (NA) with GM-CSF
b) Percentage of wound repair with 10^5 Mφ from CD patients in acute phase (A) (n=7) or in remission (R) (n=7) and differentiated (rh-GM-CSF) or not (NA) with GM-CSF

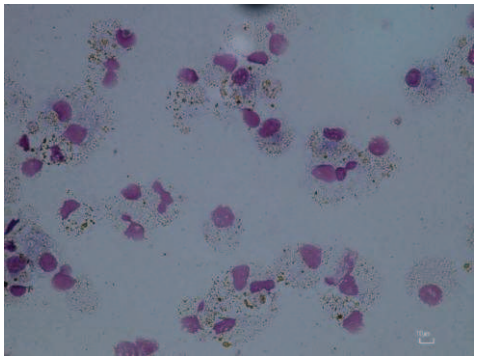
Finally, we evaluated the effect of rh-GM-CSF and anti-GM-CSF added on monocytic cells during their differentiation into Mφ, with respect to cell survival. We noted that, if anti-GM-CSF was added in the culture medium, the percentage of survival of $CD14^+$ PBMCs during their differentiation in Mφ was significantly lower than controls and the cells had a damaged phenotype. On the contrary adding rh-GM-CSF increased survival even though this difference didn't reach a statistical significance, but cells were visibly healthier and with a mature phenotype (Fig 14 and 15).

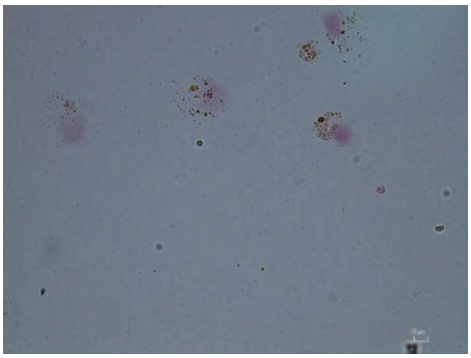


*p<0.05

Fig.14- Percentage of survival at day 5 of Mφ (10^6 Mφ per well) differentiated or not with anti-GM-CSF (n=7) or rh-GM-CSF (n=10)

a)





b)

c)

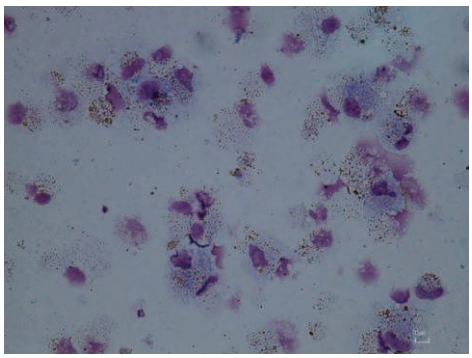


Fig.15- Giemsa stained Cytospin preparation of day 5 Mφ differentiated with anti-GM-CSF or with rh-GM-CSF

a) Control, b) anti-GM-CSF, c) rh-GM-CSF

Overall we could conclude that GM-CSF has a beneficial effect on myeloid cells, as it improves cell survival, and it can rescue the defect in wound repair that we reported for active CD Mφ. Anti-GM-CSF, on the other hand, had a negative effect on cell survival but it didn't play any role in the pro-repair function.

5. Role of Hepatocyte Growth Factor (HGF)

5.1 Is HGF implicated in our system?

We have previously demonstrated that Mφ are able to induce wound closure without cell contact, therefore we hypothesized that a soluble factor should be involved in the process.

From our previous work we observed that an increased expression of mRNA levels encoding HGF was timely associated with the GM-CSF-induced promotion of mucosal repair in the colon of DSS colitic mice[8]. Remarkably, HGF is known to be one of the soluble factors implicated in intestinal epithelial repair[21, 23, 24].

Indeed, HGF is a growth factor implicated in the repair and remodelling of the epithelium and is known to accelerate restitution[25, 26]. It has also been reported in the literature that, being also a pro-angiogenetic factor, HGF is raised in the serum of paediatric IBD patients, as well as in the serum and in the inflamed mucosa of UC patients[27].

We evaluated first if HGF could promote wound repair when added to wounded Caco.2 cells. By adding different concentrations of rh-HGF (50-150-300 pg/ml and 10 ng/ml) in our system, we observed a marked amelioration of the restitution if this molecule was added compared to controls and the healing improvement was concentration-dependent (Fig 16).

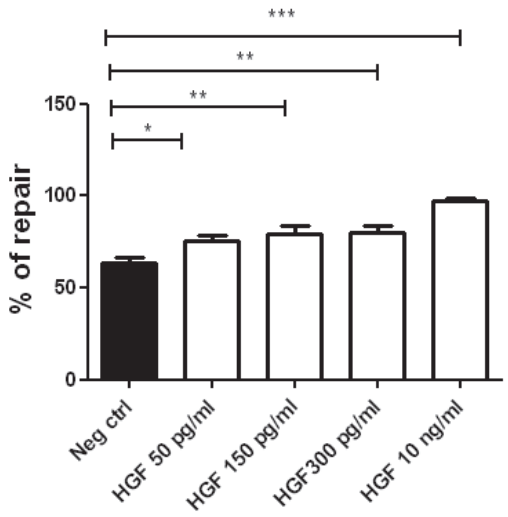


Fig.16- Percentage of repair induced by different concentrations of rh-HGF

***p<0.05**
****p<0.005**
*****p<0.0005**

We decided then to evaluate the production of HGF by Caco.2 cells and by Mφ.

We measured the concentration of HGF in the culture supernatant of the Caco.2 cell alone, of HD Mφ alone, and of co-culture wounded ECs + HD Mφ. At day 3 after wounding, Caco.2 cells could produce HGF and they produced more HGF if wounded (200 pg/ml) compared to not wounded (50 pg/ml). The production of HGF was higher in wells with wounded Caco.2 + HD Mφ (around 500 pg/ml) compared to not wounded Caco.2 + HD Mφ (300 pg/ml).

The concentration of HGF was then evaluated in the supernatant of CD Mφ at the end of differentiation (day 5 of culture) and compared with that of HD Mφ, but we couldn't find any difference between the two groups. This result was confirmed by both the dosage of protein concentration by ELISA and by quantification of mRNA expression by qPCR (Fig.17)

Interestingly, when we compared the HGF concentration at day 3 in the supernatant from co-cultures of wounded Caco+ HD or UC Mφ with the supernatant coming from wounded Caco+ CD Mφ, we found a lower concentration of HGF if CD Mφ were added than in the presence of HD Mφ or UC Mφ (Fig.18)

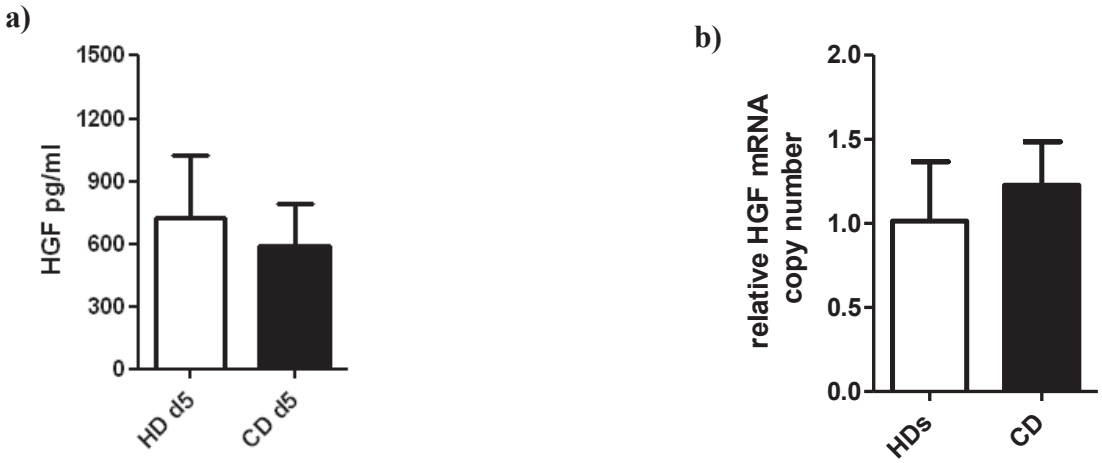


Fig.17- HGF production by HD and CD Mφ
a) HGF levels in supernatants of 10^6 Mφ at d5 of differentiation from HD (n=8) or CD patients (n=11) measured by ELISA
b) relative HGF mRNA expression of 10^6 Mφ at d5 of differentiation from HD (n=4) or CD patients (n=10) measured by qPCR

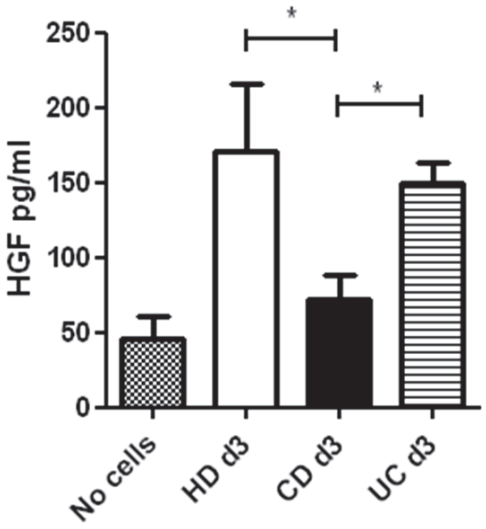


Fig.18- HGF levels in supernatants of d3 co-culture of wounded Caco.2 cells + 10^5 Mφ from HD (n=9), CD (n=13) or UC (n=4)

***p<0.05**

Overall, we could conclude that HGF can promote wound healing in our model and is produced by ECs and Mφ. Mφ secrete high levels of HGF and there is no difference in HGF production between HD and CD Mφ during their differentiation. However, if Mφ are in co-culture with wounded Caco.2 cells, CD Mφ produce less HGF than HD or UC Mφ.

The biologically active form of HGF is a heterodimer of a heavy α-chain and a light β-chain, resulting from proteolytic cleavage of the precursor. The only known high affinity receptor of HGF is c-Met, a trans-membrane receptor with tyrosine kinase activity. It is expressed by hematopoietic and epithelial cells as well as hepatocytes, fibroblasts, keratinocytes and melanocytes, and it transduces all HGF effects on target cells[25, 28].

To better understand which cells are the main producers and which are the target of HGF in our system, the mRNA expression of both *hgf* and *c-Met* was assessed by qPCR in Caco.2 cells and Mφ at d5 of differentiation from HD, RCD and ACD. Caco.2 cells expressed both *c-Met* (mean $1.88x10^5$ gene copies) and *hgf* (mean $6.97x10^5$ gene copies). It was then evaluated if the expression of these genes was different between HD Mφ and Mφ from RCD or ACD, but we couldn't find any significant difference between these groups (Fig.19).

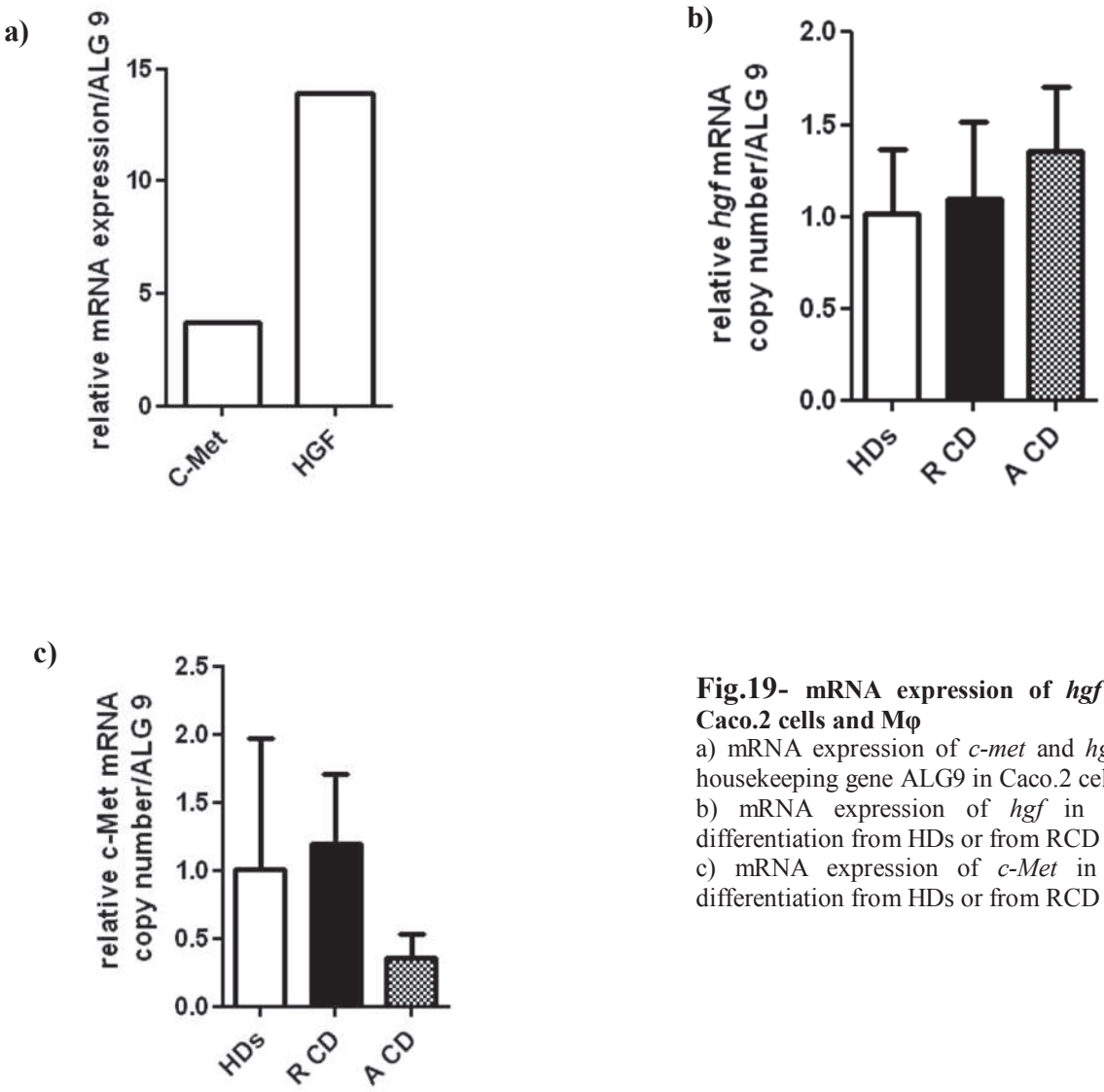


Fig.19- mRNA expression of *hgf* and *c-Met* in Caco.2 cells and Mφ
a) mRNA expression of *c-met* and *hgf* in relation to housekeeping gene ALG9 in Caco.2 cells
b) mRNA expression of *hgf* in Mφ at d5 of differentiation from HDs or from RCD or ACD (n=5)
c) mRNA expression of *c-Met* in Mφ at d5 of differentiation from HDs or from RCD or ACD (n=5)

Overall, we could conclude that Caco.2 cells express both *hgf* and its receptor *c-Met*. These two genes are also expressed by HD Mφ as well as CD Mφ, regardless of the status of the disease.

5.2 Inhibition of HGF

So far we have demonstrated that HGF is able to induce repair of Caco.2 *per se* and it is produced by both epithelial cells and Mφ. *Hgf* and its receptor *c-Met* are expressed by both Caco.2 cells and Mφ from either HDs or CD patients. Importantly, CD Mφ produce less HGF than HD Mφ at d3 of co-culture with wounded epithelial cells.

Therefore, we concluded that HGF is implicated in wound healing in our system, but we couldn't define yet if HGF was the main factor implicated in the Mφ pro-repair activity.

To test this hypothesis we evaluated the percentage of repair of wounded Caco.2 cells in the presence of an inhibitor of HGF activity. In particular we used a small molecule called PHA665752 (Tocris Biosciences), which competitively inhibits binding of ATP to the tyrosine kinase domain of c-Met. This molecule *in vitro* inhibits c-Met dependent phenotypes such as cell growth, motility, invasion and morphology, as well as phosphorylation of downstream signal transducers. *In vivo* it exhibits a dose-dependent antitumor activity in a gastric carcinoma xenograft model [29, 30].

We tested first if PHA665752 was able to inhibit HGF-induced wound healing in our model. PHA665752 was tested at 200, 400 and 600 nM for a constant HGF concentration of 10 ng/ml and a significant inhibition of wound healing was observed only for the highest concentration of PHA665752 tested (Fig. 20).

Next we evaluated if the pro-repair activity of Mφ was inhibited by PHA665752. 10^5 HD Mφ were added in our system on wounded Caco.2 cells with or without addition of PHA665752 at 600 nM on day of wounding. At day 3 it was observed a partial but significantly lower percentage of repair if the c-Met inhibitor was added in the co-culture (Fig. 21).

Overall we could conclude that HGF is one of the main molecules involved in the pro-repair activity mediated by Mφ, as its inhibition leads to a decrease of the percentage of epithelial cell repair.

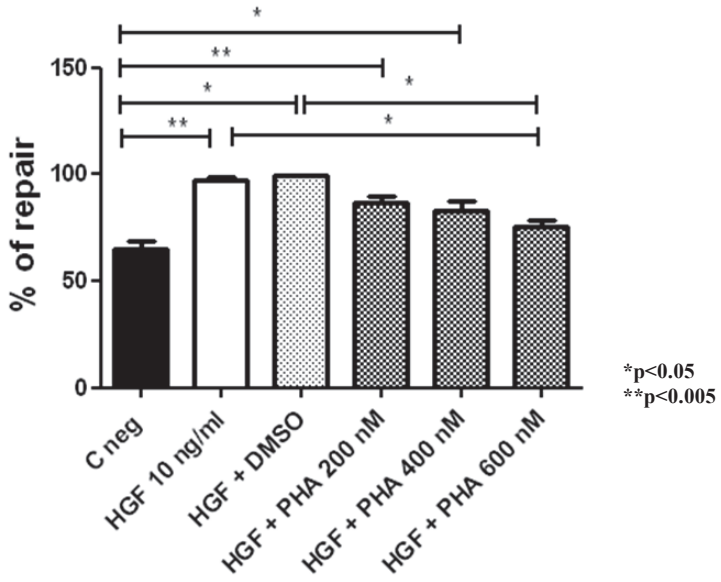


Fig.20- Inhibitory effect on wound healing of c-Met inhibitor PHA665752

This figure includes data from 4 separate experiments**.** Dimethyl sulfoxide (DMSO) was used as control as PHA is reconstituted in DMSO.

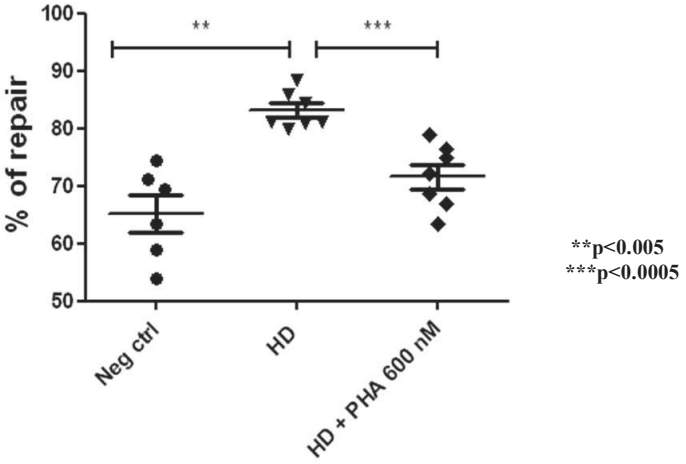


Fig.21- Inhibitory effect on the HD Mφ (n=7) pro-repair activity of c-Met inhibitor PHA665752

Each dot represents a single individual tested in a separate experiment

5.3 Co-localization of Mφ and HGF in biopsies

Given that HGF was found to have a pivotal role in the Mφ pro-repair activity in our *in vitro* system, we wondered if it was possible to show a co-localization of HGF and Mφ in human intestinal tissue.

Colonic biopsies from control patients were obtained from the CHUV Endoscopy Department, fixed in 10% buffered formalin and embedded in paraffin. Samples were then cut in serial sections of 4µm of thickness and enzymatically stained for CD68 (Mφ) and HGF. Tissue localization of CD68 was demonstrated in the same area as HGF, in particular in the instertitial tissue around the crypts (Fig. 22)

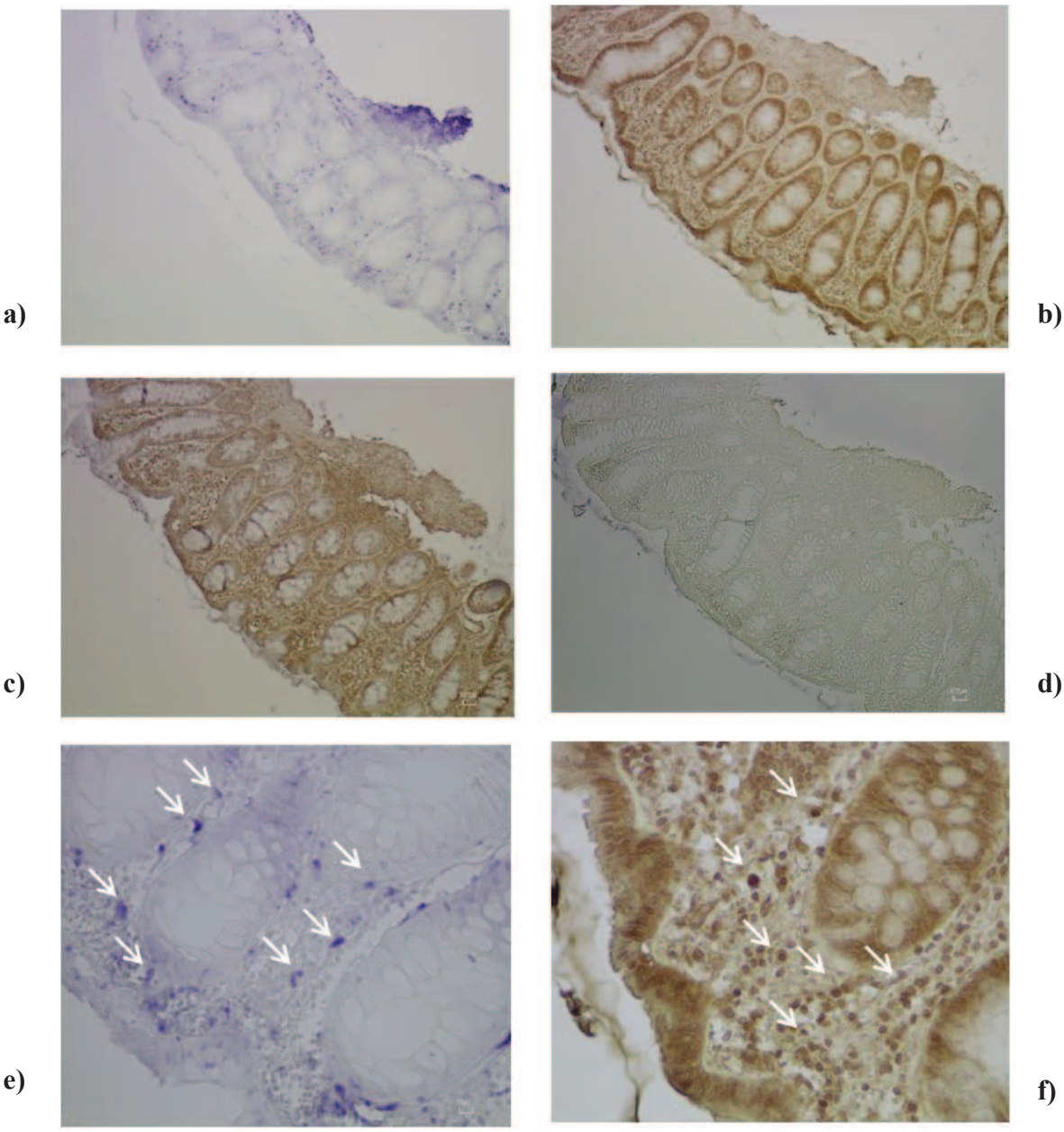


Fig.22- CD68 and HGF expression in serial sections of colonic biopsies from HD
a) CD68 at 10x magnification (scale bar 100 µm) b) HGF at 10x magnification
c) Double staining CD68-HGF 10x magnification d) Negative control at 10x magnification
e) CD68 at 40x magnification (scale bar 10 µm), white arrows pointing at positive cells
b) HGF at 40x magnification (scale bar 10 µm), white arrows pointing at positive cells

6. Role of intestinal macrophages (IMACs) in wound healing

6.1 Role of IMACs in wound healing

Intestinal macrophages represent the largest population of mononuclear phagocytes in the body and are strategically located in the subepithelial lamina propria. These cells have a double role of protecting the host against harmful pathogens breaching the epithelium and regulating the inflammatory response towards commensal gut microbiota. Thus, in a non inflamed mucosa, IMACs acquire inflammatory anergy with downregulation of innate response receptors, despite retaining a phagocytic and bactericidal activity[31-33].

IMACs have great phenotypical difference from *in vitro* differentiated Mφ as the classical monocyte markers CD14, CD16, CD11b are almost absent. CD33 was identified to be a useful recognition marker for IMACs[19].

We have previously demonstrated that HD Mφ differentiated *ex-vivo* from $CD14^+$ PBMCs exhibit a pro-repair function that is deficient in CD Mφ. We decided to investigate if the same pro-repair activity was also characteristic of IMACs. Specimens of healthy colonic mucosa were recovered from patients undergoing surgery for colon carcinoma and IMACs were isolated as described in Materials and Methods. 10^5 IMACs, which was the concentration inducing the highest percentage of repair for Mφ, were added in our wounding assay. Interestingly, a significant amelioration of repair was observed (Fig.23).

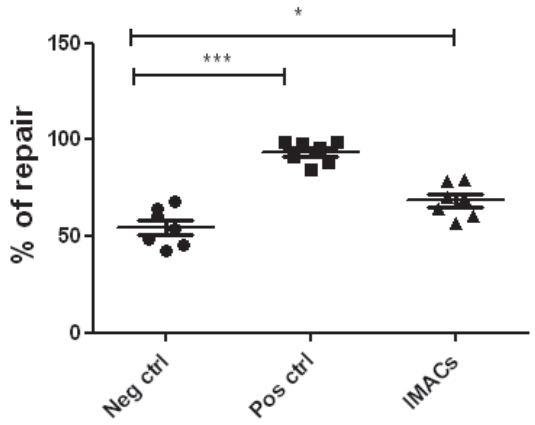


Fig.23- Amelioration of repair with HD IMACs (n=7)

Each dot represents a single HD tested in separate experiments

***p<0.05**
****p<0.0005**

Next we examined if IMACs coming from CD patients could induce the same level of repair. Due to an extreme difficulty in recruiting ACD in need of a surgical intervention, we unfortunately got hold of one single patient who had a severe ileal inflammation. The surgical specimen included both the terminal ileum and part of the caecum, which was free from inflammation. IMACs were isolated from both sites and tested in our assay. Interestingly, colonic IMACs showed a capacity to induce repair, while the ileal IMACs lacked of this ability (Fig 24)

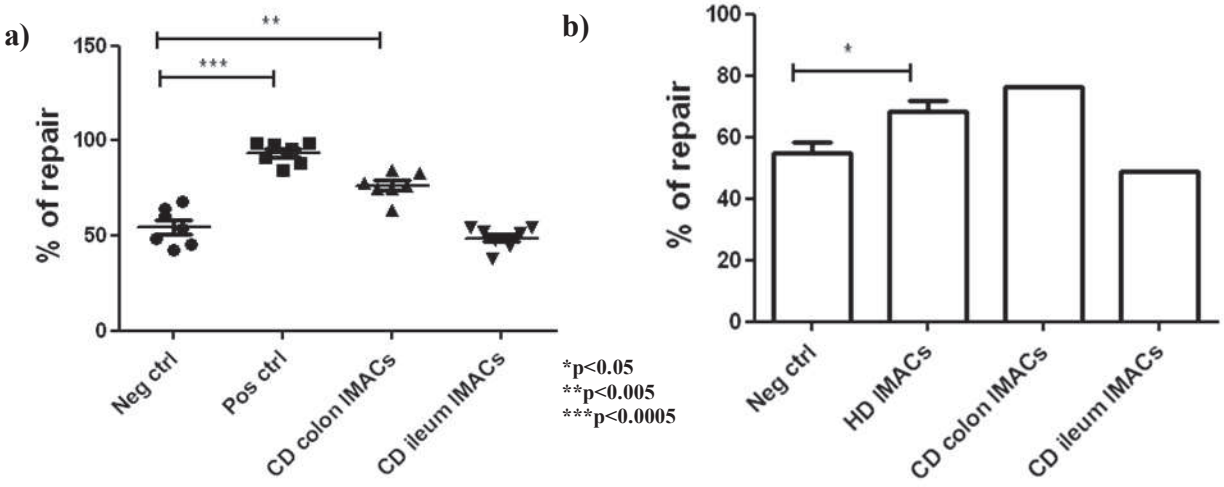


Fig.24- CD IMACs coming from inflamed ileum cannot induce wound healing
a) CD IMACs from non-inflamed colon can induce repair, while those from inflamed ileum cannot. In this experiment each dot represents a single wound.
b) Preliminary comparison between HD IMACs, non-inflamed colon CD IMACs, inflamed ileum CD IMACs.

6.2 HGF production by IMACs and its inhibition

We have demonstrated that IMACs isolated from HD are able to promote wound healing, confirming the results obtained with Mφ. We wondered if IMACs, like Mφ, promoted their repair through HGF production.

First the capacity of HD IMACs to produce HGF was evaluated. The concentration of HGF was measured by ELISA at day 3 in the supernatant from co-cultures of wounded Caco + HD

IMACs and we found that HD IMACs could produce HGF, like HD Mφ (Fig. 25)

Next we evaluated if HD IMACs' pro-repair activity was inhibited by PHA665752. 10^5 HD IMACs were added in our system on wounded Caco.2 cells with or without addition of PHA665752 at 600 nM on the day of wounding. At day 3, preliminary data with 3 HD IMACs demonstrated a partial but significantly lower percentage of repair if the c-Met inhibitor was added in the co-culture (Fig. 26).

Overall we could conclude that HGF is one of the main molecules involved in the pro-repair activity mediated by IMACs, confirming that IMACs and Mφ in our model induce wound healing through the same mechanism.

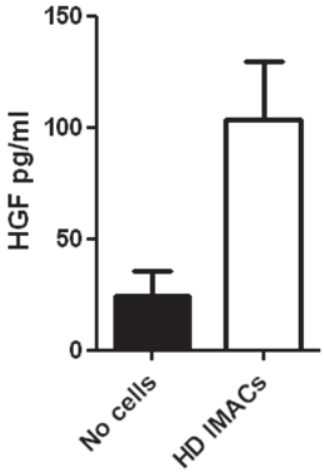


Fig.25- HGF levels in supernatants of d3 co-culture of wounded Caco.2 cells + 10^5 HD IMACs (n=5)

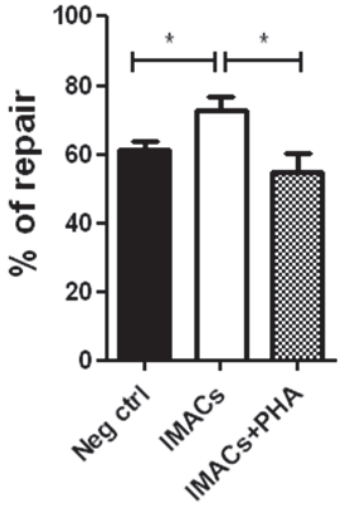


Fig.26- Inhibitory effect on the HD IMACs (n=3) pro-repair activity of c-Met inhibitor PHA665752

Discussion and Conclusion

In our work we focused our attention on the analysis of the role played by Mφ in wound healing. The rationale of our choice lies within two main reasons. First, it has been suggested that a deficiency in the innate immunity during the acute phase of the inflammation in CD patients plays an important role in the evolution of the disease[10, 11]. Secondly, our previous results in mice studies pointed out a fundamental role of GM-CSF-expanded $CD11b^+$ monocytic cells in inducing wound healing both *in vivo* and *in vitro*[8]. Mucosal healing has become one of the main targets in clinical studies, as it is associated with a better outcome of the disease. Breaches in the gut mucosa, even if minimal, induce a massive penetration of bacteria and foreign material through the bowel wall and the necessity to mount an appropriate acute response with an immediate repair of the lesion is crucial to control bacterial translocation.

In this work, an *in vitro* wound healing model has been set-up to investigate the repair function of myeloid cells. In this model we demonstrate that PBMCs are inefficient in promoting epithelial restitution. In contrast, HD PBMC-derived Mφ acquire this capacity. Most interestingly, CD Mφ exhibit a deficient pro-repair function compared to HD or UC Mφ, even though these cells are not phenotypically different. In particular there is an association between the capacity to repair and the status of the disease, as ACD Mφ have the weakest repair function. This deficient function for A CD Mφ can be rescued by adding rh-GM-CSF during the differentiation of PBMCs in Mφ. We also demonstrate that HD Mφ promote wound healing through HGF production. Indeed Mφ produce HGF and the inhibition of the HGF receptor c-Met leads to a decreased percentage of repair. Importantly, CD Mφ produce less HGF than HD or UC Mφ if in co-culture with wounded epithelial cells. In this

work it is also demonstrated that HD IMACs, as blood-derived Mφ, promote wound healing through HGF production.

The fact that PBMCs need to be differentiated in Mφ to promote wound healing indicates that acquisition of repair function requires a maturation step presumably taking place *in vivo* in the gut mucosa. In fact HD IMACs are able to induce wound healing.

Our results indicate that acquisition of the maximal repair function necessitates strong Mφ activation as obtained with HkEc, and that both HD and CD Mφ are able to respond to this microbial stimulus by increasing their repair function. Hence, impairment of repair activity in CD Mφ does not appear as an intrinsic defect in a pro-repair pathway.

On the other hand, the fact that A CD Mφ are weaker repair cells than R CD Mφ, combined to the fact that A CD Mφ can be slightly boosted by rh-GM-CSF during differentiation in contrast to R CD Mφ, supports the interpretation that A and R CD have distinct circulating myeloid cell populations[34]. Differential repair activities might also be the consequence of differential responses to the local environment that strongly differs between HD, A CD and R CD. Mφ are a very heterogeneous population influenced by both innate and adaptive signals and the cytokine *milieu* is fundamental in determining Mφ function[20].

Interestingly, UC Mφ are able to induce repair regardless of the status of the disease. This result suggests that the defect in wound healing observed for CD patients is actually specific for the disease and it is not a consequence of an indiscriminate inflammation of the bowel. Moreover, these findings are in keeping with the different histological and clinical picture of these two forms of IBD. In fact CD patients often present with transmural ulceration and fistulising disease, while in UC the inflammation is limited to the mucosa and fistulae are rare. It can be speculated that the lack of repairing abilities of CD Mφ is responsible of the phenotype observed in patients.

From our data it emerges also that soluble factors are involved in the repair process, as Mφ don't need cell contact to promote restitution. Many growth factors are known to be implicated in wound healing, such as Transforming Growth Factor- α (TGF-α), TGF-β, Keratinocyte Growth Factor (KGF), Epidermal Growth Factor (EGF), Insulin-like Growth Factor (IGF) and Hepatocyte Growth Factor (HGF) among others[23, 35, 36]. We decided to focus our attention in particular on the role played by HGF, as HGF ameliorates epithelial regeneration in both DSS and 2,4,6-trinitrobenzene sulfonic acid (TNBS) colitis models[37, 38] and HGF deficient mice exhibit impaired intestinal mucosal regeneration[39]. Moreover, HGF level was found to be increased in the serum of IBD patients[27]. In our previous work an increased expression of mRNA levels encoding HGF was timely associated with the GM-CSF-induced promotion of mucosal repair in the colon of DSS colitic mice[8]. In relation with these results obtained in the mouse model, HGF was found to play a pivotal role in the HD Mφ-induced restitution. We demonstrate that HGF is indeed a pro-repair molecule in our system and that it is produced at higher levels by HD Mφ than by CD Mφ at day 3 of co-culture with wounded epithelial cells. This finding establishes a link between HGF production by Mφ and restitution rate. However, this difference in HGF secretion is not present if the HGF level is measured at the end of Mφ differentiation, implying that the defective HGF secretion of CD Mφ is not at the level of gene transcription. This hypothesis is confirmed by our PCR data, showing no difference in the mRNA expression of both *hgf* and its receptor *c-met* in HD and CD Mφ.

The fact that CD Mφ do not secrete HGF as well as HD Mφ during the co-culture with wounded epithelial cells could be explained either by an incapacity of CD Mφ to respond adequately to danger signals or by an inefficient release of the molecule. This last hypothesis is supported by evidences in the literature demonstrating that HGF is stored in his inactive form (pro-HGF) in secretory vesicles and granules of polymorhonuclear cells (PMN),

providing a readily available stock[40]. It is therefore conceivable that, if Mφ stock HGF in vesicles as PMN do, during a tissue injury, Mφ can rapidly provide high concentration of HGF, promoting wound healing. CD Mφ could be deficient in this phase of rapid release. Segal's group provided indeed evidence that CD Mφ are deficient in secretion of pro-inflammatory cytokines and that this defect is not at the level of gene transcription. On further analysis of Mφ mRNA transcription profiles, it was identified a group of abnormally expressed genes in CD Mφ and half of them had associations with cellular secretory systems[11]. These results are even more intriguing given the known association of variants of autophagy genes with CD susceptibility and, importantly, this association was not found in UC patients[41]. A defect in autophagy means both an impaired vesicle trafficking and a dysfunction to remove intracellular organisms in Mφ, further supporting the hypothesis of Mφ deficiencies in CD patients. To test if CD Mφ secrete less HGF due to a release defect, it would be first necessary to confirm that HGF is indeed stored intracellularly and then compare HD with CD Mφ. This result can be achieved by a simple immunocytochemical staining for HGF, by an intracellular FACS analysis for HGF or by a western blot analysis of the different Mφ subcellular fractions. Once confirmed that HGF is indeed stored at intracellular level, it would be interesting to analyse the secretory capacity of CD Mφ. HGF level could be evaluated in the culture medium of HD and CD Mφ after inducing release with HkEc/LPS[42]. Lastly, a normal HGF synthesis together with reduced secretion could also mean an increased degradation of the molecule. It would be then interesting to check the functionality of the lysosomal compartment by adding lysosomal inhibitors (e.g. NH_4Cl or chloroquine, which increase lysosomal pH) to CD Mφ and then measuring the intracellular levels of HGF. If the defect is indeed a hyper-functionality of the lysosomal compartment, the HGF intracellular level in CD Mφ after treatment with lysosome inhibitors would increase to the same levels of HD Mφ.

Another hypothesis explaining the lack of CD Mφ secretion of HGF would be a defective response to danger signals, known as damage-associated molecular pattern (DAMPs). A DAMP is any molecule not normally exposed that is revealed because of a damage or injury[43]. These molecules include high mobility group box-1 (HMGB1), S100A8 (MRP8, calgranulin A), S100A9 (MRP14, calgranulin B), and heat shock protein 70 (HSP70). In particular HMGB1 is a nuclear protein known to be released passively by necrotic cells and actively secreted by immune cells, including Mφ, and enterocytes[44-46]. Its signalling is mediated by the receptor for advanced glycation end products (RAGE) and members of Toll-like family receptors. RAGE is expressed at low levels in normal tissues, it is upregulated at sites where its ligands accumulate and it is expressed by monocytic cells among others[46, 47]. The interaction of HMGB1 with its receptor RAGE mediates both pro-inflammatory and restorative effects. For instance monocytes stimulated with HMGB1 release numerous pro-inflammatory cytokines[48], and it is also demonstrated that myofibroblasts stimulated by HMGB1 migrate towards damaged regions promoting repair[49]. Given this evidence, it could be hypothesized that CD Mφ are deficient in recognizing DAMPs, as HMGB1, and therefore secrete less HGF to induce wound healing. It would be interesting to test this hypothesis by incubating HD and CD Mφ with HMGB1 and then test their respective repair capacity, or by analysing the expression of the HMGB1 receptor RAGE on the surface of HD and CD Mφ to rule out a downregulation of RAGE in CD Mφ. Given that HMGB1 is actively secreted by Mφ, it could also be postulated that this molecule works in an autocrine manner. Consequently another possibility would be that CD Mφ cannot secrete an adequate amount of HMGB1 to induce an auto-stimulation of the Mφ, eventually leading to HGF secretion. To investigate this aspect, HD and CD Mφ could be stimulated *in vitro* with pro-inflammatory cytokines and then the level of HMGB1 in the culture medium evaluated.

The importance of HGF role in our system was demonstrated by a specific inhibition of HGF with PHA665752, which competitively inhibits binding of ATP to the tyrosine kinase domain of c-Met. This molecule was found to be more than 50 times more selective for c-Met compared with a panel of other tyrosine and serine-threonine kinases[30]. Indeed, the percentage of HD Mφ-induced wound healing was significantly lower if PHA665752 was added in our system. This result demonstrates that HGF is one of the major factors involved in the Mφ mediated pro-repair activity.

In our work we also demonstrate a co-localization of HGF and Mφ in the lamina propria of human colonic biopsies, suggesting that Mφ could be a HGF source also *in vivo*. Unfortunately, technical issues, namely the autofluorescence of human colonic tissue, didn't allow us to proceed with more precise techniques (i.e. immuno-fluorescent staining), which would have allowed us to demonstrate the presence of HGF and Mφ markers in the same cells.

Initially, our results have investigated the repair function of *in vitro* derived Mφ. However, it is established that both monocytes and *in vitro* derived Mφ are phenotypically and functionally different from intestinal macrophages (IMACs) [19, 31-33]. Therefore we decided to investigate the pro-repair function of IMACs isolated from the healthy intestinal mucosa of patients undergoing surgery for a colon carcinoma. These IMACs were able to induce repair, to produce HGF, and PHA665752 inhibited their activity, confirming the results obtained with HD Mφ. IMACs are undoubtedly a good tool to investigate the healing function of macrophages, being the cells actually present in the mucosa. It has however to be considered that surgical specimens are not easy to collect. Moreover the IMACs isolation procedure includes enzymatic digestions and mechanical separations that can compromise cell functionality. On the opposite, blood samples are readily available and blood-derived Mφ have been widely used in the literature to study characteristic of intestinal macrophages[10, 11].

Our aim was to compare the wound healing function of HD IMACs with IMACs from CD patients, but, due to the extremely low number of surgeries for CD, we obtained a single patient only. These very preliminary data are nevertheless very intriguing, as CD IMACs from non-inflamed caecum were able to induce healing, while CD IMACs from inflamed ileum could not. To confirm these data we would need first of all to demonstrate that HD IMACs from ileal mucosa can induce repair, as so far we have tested HD colonic IMACs only. Some evidence identified differences between ileal and colonic macrophages in non inflamed mucosa. Normal colonic macrophages were larger and more strongly positive for acid phosphatase and non-specific esterase than macrophages in terminal ileum[50]. However there was no difference between ileal and colonic macrophages in their ability to undergo a respiratory burst[51]. In future studies we would need to accumulate more CD IMACs from both colonic and ileal sites and compare them with corresponding HD IMACs. If indeed the ability to induce repair is not dependent on the site of macrophage-extraction, it could be speculated that is then the cytokine environment responsible to modulate IMACs phenotype and functionality. It is reasonable to imagine that in a severely inflamed mucosa, as to necessitate a surgical intervention, the IMACs would shift to a more pro-inflammatory macrophage phenotype. It has already been demonstrated that in CD there is an increased recruitment of blood-derived $CD14^+$ monocytes in the mucosa, with upregulation of co-stimulatory molecules (CD80 and CD 86) and TREM-1[52-54]. It would be interesting to test the pro repair ability of IMACs at the very beginning of the acute phase of the inflammation. A lack of healing capacity would support the hypothesis of an innate immunodeficiency in CD.

What we have observed so far is that *in vitro* generated CD Mφ, and in particular ACD Mφ, cannot repair as well as HD Mφ. This defect seems to be related to a lower production of HGF in an acute situation (i.e. the contact with a wounded epithelial layer). It can be speculated that at the very early stage of the acute phase, when the first breaches in the

mucosa appear and foreign material start penetrating through the bowel wall, CD resident macrophages are unable to promptly react to the new acute situation. Segal et al. demonstrated that CD macrophages have a weak inflammatory response, with low pro-inflammatory cytokine production, likely leading to low neutrophil recruitment and consequent impaired bacterial clearance[10]. We could add that CD macrophages are also deficient in promoting a rapid mucosal healing, probably due to incapacity to secrete adequate amounts of HGF. This slow repair of the mucosa causes an even wider penetration of microorganisms, which will eventually lead to the activation of an adaptive immune response and a chronic inflammation.

Given the observed important role of HGF in inducing wound repair, it could be tempting to propose HGF as a potential therapy for Crohn's disease, especially in light of the increasing importance of inducing mucosal healing in CD patients. Some issues are nevertheless to be taken into account before proposing HGF in a clinical trial. First, HGF is a pro-angiogenetic and an epithelial growth factor, therefore potentially increasing the risk of neoplastic transformation. Administration of HGF intraperitoneally in a rat model of IBD induced an amelioration of the disease and reduced ulceration score without increasing epithelial mitotic rate[55]. A preliminary double-blind, placebo-controlled study was performed to investigate the potential therapeutic role of Growth Hormone (GH) in CD. The study demonstrated an amelioration in the CDAI after four months of treatment and there was no evidence of an increase in cancer development (two patients out of 15 in GH group were diagnosed with cancer following investigations for symptoms already present before being included in the study)[56]. Despite these encouraging results, a cautious approach is required in particular in the context of a chronic disease which usually requires long and repeated cycles of therapy. Secondly, the route of HGF administration should be chosen carefully. Intravenous administration lacks a precise targeting of ulcerated regions while a topical administration

would be preferable, through enemas, suppositories or endoscopically administered injections. A Japanese group recently demonstrated a significant repair of injured rectal mucosa in rat experimental colitis treated with HGF rectal enemas[57].

Our results also support the theory of CD as an innate immune-deficiency. If future evidence confirms this hypothesis, it may be interesting to consider a therapy aiming at the stimulation of the innate acute response or promoting the bone-marrow production of pro-repair myeloid precursors.

In conclusion, despite being aware that our results are based on *in vitro* experiments, we were able to detect a deficient function in CD Mφ to promote wound healing that, to our knowledge, has never been reported before. Our data are in keeping with the current tendency in the literature of considering CD as an innate immune-deficiency. We believe that more investigations in this direction would contribute to elucidate further the pathogenesis of Crohn's disease and possibly would lead to a reassessment and an improvement of current treatment options.

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