ORIGINAL ARTICLE

MiR-155 modulates the inflammatory phenotype of intestinal myofibroblasts by targeting SOCS1 in ulcerative colitis

Surajit Pathak[1,2], Alessia Rosaria Grillo[1,2], Melania Scarpa[3], Paola Brun[2], Renata D'Incà[1], Laura Nai[1,2], Antara Banerjee[1], Donatella Cavallo[2], Luisa Barzon[2], Giorgio Palù[2], Giacomo Carlo Sturniolo[1], Andrea Buda[1] and Ignazio Castagliuolo[2]

Abnormal levels of microRNA (miR)-155, which regulate inflammation and immune responses, have been demonstrated in the colonic mucosa of patients with inflammatory bowel diseases (IBD), although its role in disease pathophysiology is unknown. We investigated the role of miR-155 in the acquisition and maintenance of an activated phenotype by intestinal myofibroblasts (IMF), a key cell population contributing to mucosal damage in IBD. IMF were isolated from colonic biopsies of healthy controls, ulcerative colitis (UC) and Crohn's disease (CD) patients. MiR-155 in IMF was quantified by quantitative reverse transcription-PCR in basal condition and following exposure to TNF-α, interleukin (IL)-1β, lipopolysaccharide (LPS) or TGF-β1. The effects of miR-155 mimic or inhibitor transfection on cytokine release and suppressor of cytokine signaling 1 (SOCS1) expression were assessed by enzyme-linked immunosorbent assay and western blot, respectively. Regulation of the target gene SOCS1 expression by miR-155 was assessed using luciferase reporter construct. We found that miR-155 was significantly upregulated in UC as compared with control- and CD-derived IMF. Moreover, TNF-α and LPS, but not TGF-β1 and IL-1β, significantly increased miR-155 expression in IMF. Ectopic expression of miR-155 in control IMF augmented cytokines release, whereas it downregulated SOCS1 expression. MiR-155 knockdown in UC-IMF reduced cytokine production and enhanced SOCS1 expression. Luciferase reporter assay demonstrated that miR-155 directly targets SOCS1. Moreover, silencing of SOCS1 in control IMF significantly increased IL-6 and IL-8 release. In all, our data suggest that inflammatory mediators induce miR-155 expression in IMF of patients with UC. By downregulating the expression of SOCS1, miR-155 wires IMF inflammatory phenotype.

INTRODUCTION

Inflammatory bowel diseases (IBD) refer to idiopathic, chronic, relapsing, inflammatory conditions arising from an underlying immunological deregulation triggered by currently unknown factors; Crohn's disease (CD) and ulcerative colitis (UC) are the two major clinical forms of IBD.[1,2] Mounting evidence suggests that mesenchymal cells, such as intestinal fibroblasts and myofibroblasts (IMF), are actively involved in the inflammatory process of the intestinal mucosa.[3] Stable alterations in their phenotype and function during persistent inflammation can regulate the transition to chronic gut inflammation by promoting tissue destruction and supporting the recruitment, retention and activation of immune cells by producing a diverse array of cytokines.[4] However, mechanistic data, as

how activated IMF maintain their inflammatory phenotype, are limited.

MicroRNAs (miRNA) are small non-coding RNAs that primarily bind to the 3′-untranslated region (UTR) of their specific target mRNAs, thus preventing protein synthesis.[5] Intracellular signaling pathways induce or repress specific miRNA to switch gene expression programs of cells, strongly influencing signal transduction pathways and cellular phenotype.[6,7] Deregulated miRNA expression has been linked to a variety of disorders such as autoimmune diseases, cancers and chronic inflammatory pathologies, such as IBD.[8–11] MiR-155 is a typical multifunctional miRNA involved in hematopoiesis, inflammation and immune responses,[12] whose expression is induced by inflammatory cytokines and toll-like

[1]Department of Surgery Oncology and Gastroenterology DISCOG, University of Padova, Padova, Italy; [2]Department of Molecular Medicine, University of Padova, Padova, Italy and [3]Oncological Surgery Unit, Veneto Institute of Oncology IOV-IRCCS, Padova, Italy
Correspondence: Professor I Castagliuolo, Department of Molecular Medicine, University of Padova, Via Gabelli, 63, 35121 Padova, Italy.
E-mail: ignazio.castagliuolo@unipd.it
Received 9 September 2014; revised 30 December 2014; accepted 12 January 2015

receptor ligands.[13] Therefore, it is not surprising that abnormal levels of miR-155 have been observed in the colonic mucosa of IBD patients.[14–17] However, the cellular sources of miR-155 in the colonic mucosa and its contribution to disease pathophysiology have not been investigated.

In the present study, we found that miR-155 is significantly upregulated in IMF isolated from UC patients but not from CD patients. Its expression in IMF is regulated by pro-inflammatory signals, such as TNF-α and lipopolysaccharide (LPS), but not by pro-fibrogenic mediators, such as TGF-β1. Moreover, we showed that the overexpression of miR-155 in control IMF recapitulates the inflammatory phenotype of UC-derived IMF, whereas miR-155 knockdown in UC-derived IMF corrects their pro-inflammatory phenotype. Furthermore, we found that miR-155 directly targets the suppressor of cytokine signaling 1 (SOCS1), whose expression is significantly downregulated in UC-derived IMF. Altogether, these results provide a better understanding in UC pathophysiology and identify a potential new molecular target to design anti-inflammatory therapies.

MATERIALS AND METHODS

Patients
We collected biopsies from patients with UC ($n = 8$) or CD ($n = 8$) undergoing colonoscopy for their ordinary follow-up. Biopsies were obtained from macroscopically normal colonic mucosa, and histological analysis of an immediately adjacent area confirmed the absence of active inflammatory damage. In Table 1, patients' clinical details are summarized. Controls were biopsies collected from subjects undergoing colonoscopy for cancer screening ($n = 8$), showing no endoscopic signs of disease (Table 1). All study procedures were complied with the principles of the amended Declaration of Helsinki and were approved by the Ethical Committee of the University Hospital of Padua. Each patient was provided with detailed information about the study aims and protocol and gave their written informed consent before enrollment. Biopsies were obtained from the sigmoid region, at 8–10 cm from the anus. In UC patients, disease activity was scored using the Schroeder (a.k.a. Mayo) endoscopic

Table 1 Characteristics of patients

	Crohn's disease	Ulcerative colitis	Control
Gender (F/M)	4/4	5/3	4/4
Mean age (years)	39	42	55
Age range (years)	28–64	24–71	27–69
Localization of previous activity	Colon 8	Pancolitis 2 left colitis 6	NA
Activity	CDAI < 150	Truelove–Witts < 5	NA
Disease duration range (years)	2–15	3–18	NA
Medications (%)	5 ASA 75%	5 ASA 100%	NA
	Steroids 0%	Steroids 0%	
	Azathioprine 0%	Azathioprine 0%	
	Infliximab 0%	Infliximab 0%	
	None 25%	None 0%	

Abbreviations: CDAI, Crohn's disease activity index; F, female; M, male; NA, not applicable; 5 ASA, 5-aminosalicylic acid.

scoring system,[18] whereas CD activity was determined using the Crohn's disease activity index.[19]

Isolation, cultivation and characterization of human IMF
For IMF isolation, colonic biopsies were immediately placed in ice-cold Roswell Park Memorial Institute (Gibco, Life Technologies Inc., Grand Island, NY, USA). Mucus was removed by washing in phosphate-buffered saline containing 0.04% dithiotreitol (Applichem Gmbh, Darmstadt, Germany) for 10 min. The epithelial layer was removed by incubation with 10 mm EDTA for 30 min at 37 °C. Tissues were washed in Roswell Park Memorial Institute containing 1% penicillin/streptomycin and subjected to enzymatic digestion in 0.05% w/v collagenase type IV Clostridium histolyticum (Sigma-Aldrich, St Louis, MO, USA) and 4 mg ml^{-1} DNase (Calbiochem, San Diego, CA, USA) dissolved in Roswell Park Memorial Institute for 30 min at 37 °C. Tissues were washed three times in Roswell Park Memorial Institute and resuspended in Dulbecco's modified Eagle's medium supplemented with 20% v/v heat inactivated fetal bovine serum (Gibco), 2 mm L-glutamine, 0.1 mm non-essential amino acids, 1 mm sodium pyruvate, 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 0.25 µg ml^{-1} amphotericin B (all from Gibco, and indicated as complete medium). Tissues were seeded in culture plates in a controlled atmosphere of 5% CO$_2$ and 37 °C for 48 h without changing the medium to allow IMF to migrate from the tissue and adhere to plastic.[20] Cells were characterized after four passages by immunocytochemistry using mouse monoclonal anti-α-SMA and anti-desmin antibodies (dilution 1:300) (all from Sigma-Aldrich) and used for experiments up to passage 8.

MiRNA isolation and quantification by quantitative RT-PCR
IMF (5×10^5) were seeded in six-well plates and after 48 h fresh medium containing 5% v/v, fetal bovine serum was added to the cells. Total miRNA was isolated after an additional 24 h using Qiagen miRNA isolation kit (Ambion, Austin, TX, USA) according to the manufacturer's protocol. miRNA was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Expression level of mature miR-155 was determined with the TaqMan MicroRNA Reverse Transcription kit, and the TaqMan MicroRNA Assay for miR-155 and RNU6b small nuclear RNA (used as internal control) purchased from Applied Biosystems (Foster City, CA, USA). All experimental procedures were performed according to the manufacturer's protocols. In total, 10 ng of miRNA were reverse transcribed in a final volume of 15 µl, and quantitative reverse transcription-PCR was carried out on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) in a final volume of 20 µl. All quantitative reverse transcription-PCR reactions were run in duplicate. The expression of miR-155 was calculated using the formula $2^{-\Delta\Delta CT}$, where $\Delta CT = (CT_{miRNA} - CT_{miRNAcontrol})$ and CT (that is, threshold cycle) indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold.[21]

Ectopic expression and knockdown of miR-155 in IMF
To examine the effect of miR-155 overexpression, pre-miRNA155 (PM12601) and pre-miRNA scramble control (AM 17150) (Invitrogen Corp.) were transfected into control IMF. Similarly, to examine the effect of miRNA155 knockdown, the anti-miR-155 inhibitor (AM12601) and anti-miR inhibitor negative control (AM 17010, all from Invitrogen) were transfected into UC-derived IMF. IMF (4×10^4 cells/9.6 cm^2) were cultured for 24 h, washed, placed in serum-free Dulbecco's modified Eagle's medium without antibiotics and

transfected. Transfection conditions and efficiencies were established in preliminary assays measuring the level of the target gene *Hmga2* following IMF transfection with Let7c-miRNA inhibitor. IMF transfection using 6 μl Lipofectamine 2000 (Invitrogen Corp.) with 200 pmol of anti-mir let-7c miRNA inhibitor was the most efficient combination with a sevenfold increase in *Hmga2* mRNA level (data not shown) after 24 h. These conditions were then used for all miRNA transfection experiments. IMF were harvested after 24 h and used for the specified experiments.

Effect of pro-inflammatory mediators on miR-155 in IMF
IMF isolated from normal or UC patients were seeded (5×10^5 per 9.6 cm^2) and after 24 h the culture medium was changed. Then rhTNF-α, rhIL-1α, rhTGF-β1 (all at 10 ng ml^{-1}) or bacterial LPS (1 μg ml^{-1}, Sigma-Aldrich) was added to the culture medium. Cells were harvested after 24–72 h, and miR-155 quantified as described above.

SOCS1 silencing
Specific SOCS1 Silencer Select siRNA (s16468 and s16470) and Silencer Select-negative control siRNA no. 1 were purchased from Invitrogen and 20 μmol l^{-1} stock solutions were prepared in diethyl pyrocarbonate water. None of them has any significant homology to human genes (Invitrogen-held data). SiRNA were transfected when IMF reached 50% confluence. Lipofectamine 2000 and Silencer Select siRNA (80 nmol l^{-1}) were used in a total transfection volume of 2 ml per well. After 8 h, 2 ml of culture medium were added to each well. Negative controls, including non transfected IMF and cells incubated with either Lipofectamine 2000 alone or negative control siRNA, were established in parallel. IMF were cultured for an additional 2 days before performing the experiments.

Western blot analysis
IMF treated as specified were washed with ice-cold phosphate-buffered saline and then homogenized in extraction buffer (100 mM KCl, 3 mM NaCl, 3.5 mM MgCl$_2$ and 10 mM HEPES; pH 7.4) containing 1% v/v Triton X-100, 1% v/v Protease Inhibitor Cocktail Set I (Calbiochem) and phosphatase inhibitors (25 mM NaF and 10 mM Na$_3$VO$_4$). After 45 min on ice, cells were centrifuged ($1500 \times g$ for 5 min at 4 °C) to remove cellular debris. Then the supernatant designated as the total protein fraction was collected and the protein concentration was determined by the bicinchoninic acid assay. In total, 20 μg proteins for each condition were fractionated through sodium dodecyl sulphate-polyacrylamide gel electrophoresis gel and transferred to nitrocellulose membranes (Trans-Blot Transfer Medium, Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked in phosphate-buffered saline containing 0.1% Tween-20 with 5% (w/v) non-fat dry milk or 5% bovine serum albumin for 1 h at room temperature and then incubated with rabbit anti-SOCS1 (1:1000, Cell Signaling Technology, Danvers, MA, USA) and mouse anti-β-actin antibody (1:5000, Sigma-Aldrich), respectively, overnight at 4 °C. After extensive washing, membranes were incubated for 1 h with proper secondary horseradish peroxidase -conjugated antibody (1:2000, all from Sigma-Aldrich). Immunocomplexes were visualized using enhanced chemiluminescence western blotting detection reagents (Pierce, Milan, Italy). Images were acquired using a VersaDoc imaging system (Bio-Rad Laboratories) and a densitometric quantification measurement of the band intensity was performed using ImageJ software.

Secretion of inflammatory cytokines
IMF isolated from normal subjects or UC patients were seeded in 24-well plates (4×10^4 cells per well) and, after 24 h, were placed in serum-free Dulbecco's modified Eagle's medium without antibiotics. When indicated cells were transfected as described above with either pre-miRNA (PM12601) or anti-miRNA-155 (AM12601) or SOCS1 Silencer Select siRNA (s16468 and s16470) and then cultured for additional 48 h in culture medium. Medium was then refreshed in the presence or absence of LPS (1 μg ml^{-1}). Culture medium was collected after 24 h and used to quantify interleukin (IL)-6 and IL-8 release by enzyme-linked immunosorbent assay (Immunotools).

MiR-155 target validation
Through TargetScan, a bioinformatics prediction program that can identify hypothetical mRNA-binding-specific miRNA, we identified SOCS1 as a potential target of miR-155. Genomic DNA extracted from peripheral blood mononuclear cells was used as template to amplify a fragment of the 3′-UTR of SOCS1, which contains the putative miR-155-binding sequence. The following primers were used: forward 5′-gagaGAGCTCAGATTTGACCGGCAGCGCCC-3′, reverse 3′-gagaTCTAGATGCACAGCAGAAAAATAAAGCCAGA-5′. The genomic fragment was cloned into a luciferase reporter construct (pmir-GLO, Promega, Madison, WI, USA). A control reporter plasmid (pmir-GLO-3′UTR Mut) with a deletion of seven nucleotides corresponding to miR-155 seed sequence (GCATTA) was constructed by site-directed mutagenesis using forward primer 5′-gagaccg gcagcgcccgccgtgcacgcactgggatgccgtgttat-3′ and the same reverse primer was used in wild-type reporter. Then, each reporter plasmid was co-transfected into HEK 293A cells in 24-well plates together with Firefly luciferase plasmid pGL3 (Promega) and pre-miR-155 or pre-miR scramble as control. The luciferase activity in cell extracts was assayed 48 h after transfection using a Dual-Luciferase Reporter Assay System (Promega) and a 96-well plate reader. All experiments were repeated at least three times.

Statistical analysis
Statistical analysis was performed using the GraphPad Prism 3.03 (San Diego, CA, USA). Unpaired groups of values were compared according to the non-parametric Mann–Whitney test. Comparison of more than two groups of data was performed by Kruskal–Wallis test. Data were considered statistically significant for $P < 0.05$.

RESULTS
MiR-155 is upregulated in UC-derived IMF
The expression of miR-155 was determined in human primary IMF using quantitative reverse transcription-PCR. As shown in Figure 1a, miR-155 is significantly upregulated in UC-derived IMF as compared with cells isolated from CD and control colonic mucosa. These results suggest that IMF are cellular sources of miR-155 in the colonic mucosa of UC patients and may contribute to disease pathophysiology.

TNF-α and bacterial LPS upregulate miR-155 expression in IMF
IMF phenotype results from the elaboration of a variety of stimuli arising from the extracellular milieu, thus we investigated the influence of different soluble mediators involved in the development, recurrence and exacerbation of the inflammatory process in UC on miR-155 expression. As shown in

4

Figure 2, although TGF-β1 significantly influences IMF physiology,[22,23] it failed to modify miR-155 level in IMF isolated from control subjects (Figure 2a) and had only transient effects on UC-derived IMF (Figure 2b). However, an inflammatory cytokine such as TNF-α and bacterial LPS significantly modulated miR-155 level in IMF. Thus, bacterial LPS and TNF-α increased miR-155 level following 72 h exposure in control-derived IMF and further upregulated miR-155 level in UC-derived IMF. On the other hand, IL-1β had no significant effects on either control- and UC-derived IMF.

MiR-155 promotes a pro-inflammatory phenotype in IMF
In line with previous reports,[24–26] UC-derived IMF show at baseline an activated phenotype as demonstrated by the increased constitutive release of the pro-inflammatory cytokines IL-6 and IL-8 (Figures 3a and b). Indeed, over-expression of miR-155, obtained by transfecting IMF with pre-miR-155, augmented IL-6 in control IMF to a level comparable to LPS (Figures 3c and d). Furthermore, normalizing miR-155 level in UC-derived IMF by transfecting an anti-miR-155 significantly reduced IL-6 and IL-8 release (Figures 3e and f).
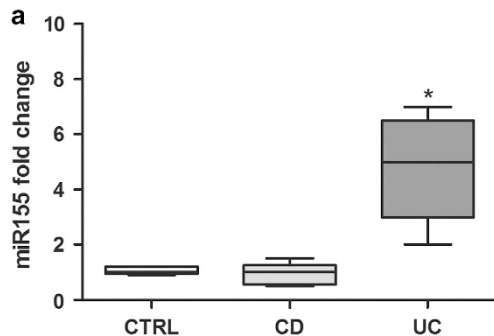

Figure 1 Increased miR-155 expression in UC-derived IMF. (a) miR-155 levels were quantified by real-time qPCR, and data normalized against U6B RNA expression in IMF isolated from mucosa of control, CD and UC patients (n = 8 each group). Data are presented as box–whisker plots (box, 25–75%; whisker, 10–90%; line, median); *P < 0.01 vs control- and CD-derived IMF.

MiR-155 and SOCS1 expression are inversely correlated in UC-derived IMF
To select potential target genes of miR-155, we used TargetScan prediction program (www.targetscan.org) to identify genes that harbored miR-155 target sites in the 3′-UTR of their mRNA. Because miR-155 influenced the inflammatory phenotype of IMF, we focused on putative candidates known to be associated with control of inflammatory processes. We identified SOCS1 as a potential miR-155 target in IMF (Figure 4a). Interestingly, recent studies revealed that SOCS1 deficiency exacerbates intestinal inflammation.[27,28] Moreover, we found that SOCS1 protein level was significantly lower in UC-derived IMF than in control-derived IMF (Figure 4b). These findings suggest an inverse relationship between miR-155 and SOCS1 expression because increased miR-155 expression is associated with the downregulation of SOCS1 in UC-derived IMF.

MiR-155 inhibits SOCS1 expression by directly targeting its 3′-UTR
To demonstrate a direct interaction between miR-155 and SOCS1, we performed luciferase reporter assays in HEK 293A cells co-transfected with reporter plasmids carrying a fragment of the 3′-UTR region of SOCS1 gene and pre-miR-155 to overexpress this miRNA. As shown in Figure 5a, miR-155 mimic significantly reduced the luciferase activity of the reporter fused with the 3′-UTR of human SOCS1 but not of the construct with a mutated 3′-UTR of SOCS1 in which the seven nucleotides of the miR-155 seed-matched sequence were deleted (3′-UTR Mut). Furthermore, transfection of miR-155 mimic in control IMF significantly reduced SOCS1 basal expression as shown by western blot analysis (Figure 5b), whereas anti-miR-155 transfection normalized SOCS1 level in UC-derived IMF (Figure 5c). These results indicate that SOCS1 is a direct target of miR-155 in IMF.

SOCS1 influences IMF inflammatory phenotype
As SOCS1 is a known feedback inhibitor of inflammation, we asked whether SOCS1 could regulate the inflammatory phenotype of IMF. We initially determined the ability of specific SOCS1 siRNAs to knockdown SOCS1 in control IMF. As
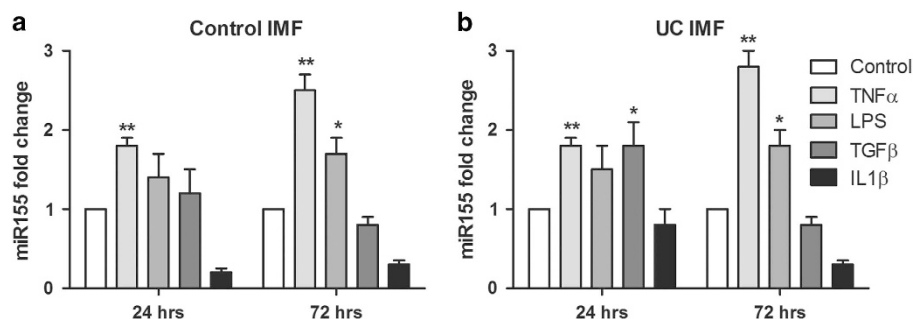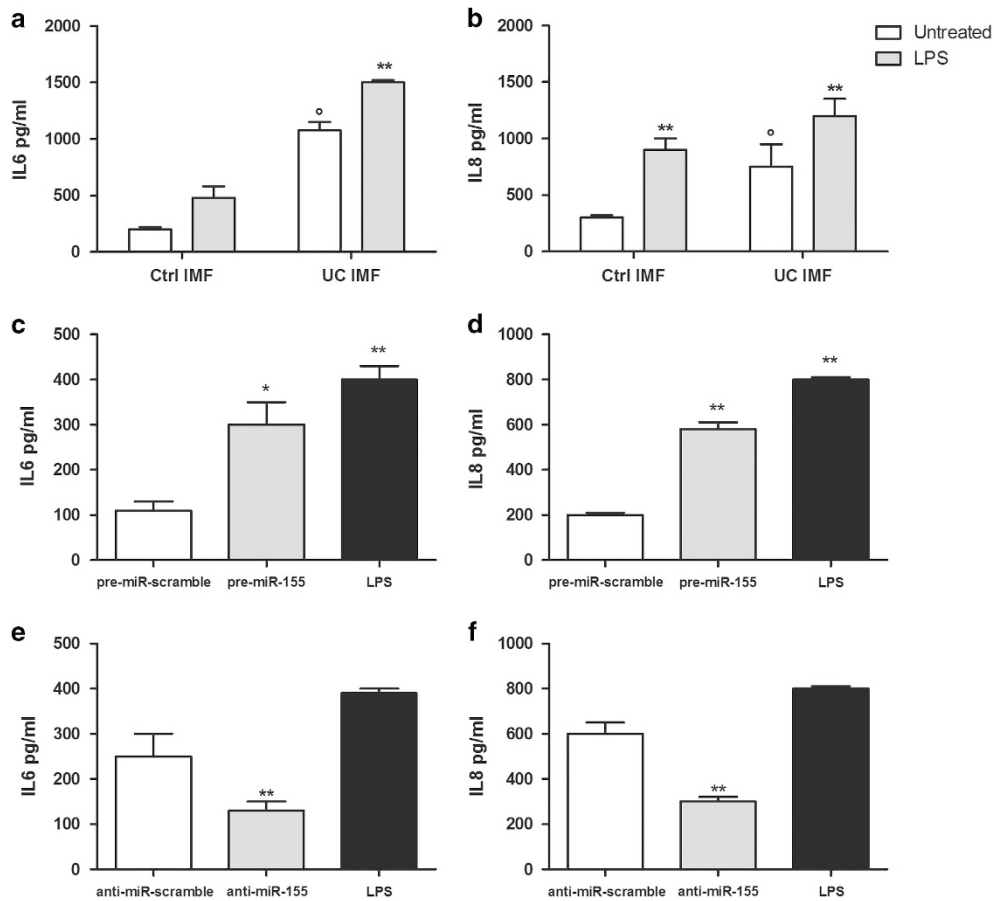

Figure 2 TNF-α and bacterial LPS upregulate miR-155 expression in IMF. Control (a) and UC-derived (b) IMF were exposed for 24–72 h to either TNF-α, LPS, TGF-β1 or IL-1β. MiR-155 levels were quantified by real-time qPCR. Data were normalized against U6B RNA expression. Data are presented as mean ± s.e.m. (n = 3). **P < 0.01 and *P < 0.05 vs respective control.

Figure 3 MiR-155 modulates the inflammatory phenotype of IMF. (**a**) and (**b**) Control- and UC-derived IMF were incubated in medium alone or LPS (1 μg ml-1) for 24 h ($n=3$ for each line with triplicate determinations); IL-6 and IL-8 levels in the supernatants were determined by ELISA. Data are expressed as mean ± s.e.m. ** indicates $P < 0.01$ and *$P < 0.05$ vs respective control, ° indicates $P < 0.01$ vs control IMF. (**c**) and (**d**) Control-derived IMF were transfected with pre-miR-155 to obtain miR-155 overexpression, and after 48 h cells and culture media were collected; IL-6 and IL-8 release was determined by ELISA. Data are expressed as mean ± s.e.m. ($n=3$ with duplicate determinations). ** indicates $P < 0.01$ and *$P < 0.05$ vs pre-miRNA scramble. (**e**) and (**f**) UC-derived IMF were transfected with anti-miR-155 to obtain miR-155 knockdown and after 48 h cells and culture media were collected. IL-6 and IL-8 release was determined by ELISA ($n=3$ with duplicate determinations). **$P < 0.01$ and *$P < 0.05$ vs respective control.

shown in Figures 6a and b, transfection with specific SOCS1 siRNAs reduced SOCS1 levels by ~70% after 48 h, whereas negative control siRNA was ineffective. As shown in Figures 6c and d, SOCS1 knockdown in control-derived IMF caused a significant increase in basal and LPS stimulated IL-6 and IL-8 release, supporting its regulatory role in the development of an inflammatory phenotype of IMF.

DISCUSSION

In IBD, the deregulated activity of several immune and non immune cell populations generates, amplifies and maintains tissue damage.[29] However, the specific gene expression profile and the signaling pathways altered in cellular populations relevant to IBD pathophysiology are scarcely known. In this study, we report that miR-155 has an important role in the aberrant phenotype of IMF in UC. In addition, we demonstrate that SOCS1 is targeted by miR-155 and that restoring miR-155 levels in UC-IMF recovers their phenotype. These findings are significant to the IBD field for several reasons. First, they

provide an miRNA expression signature of the inflammatory phenotype of IMF in UC. Second, they establish a direct link between miR-155 and IMF pro-inflammatory behavior in UC. Third, they identify IMF as a cellular population, contributing to the inadequate SOCS1 expression during colitis. Finally, our data ascertain SOCS1 as a direct target of miR-155 in primary human IMF.

MiRNAs are increasingly recognized as an important element in the development and function of the innate and adaptive immune system, and changes in miRNA expression profile are described in many immune-related diseases.[30] Elucidating the role of miRNA in IBD represents a new frontier to advance our knowledge of disease mechanisms, and to develop new diagnostic and therapeutic tools as miRNA controls basic biological functions such as proliferation, apoptosis and cell differentiation.[31] Previous studies have reported that the expression of several miRNA is altered in the intestinal mucosa during inflammatory disorders, including UC.[14–17,32] However, our study identified IMF as a cellular

source of miR-155 deregulation in the colonic mucosa of UC patients, in addition to B and T lymphocytes and macrophages following exposure to pathogens.[33,34] Indeed, the key role of

a

3' UTR of Socs1   5' CGCCGUGCACGC----AGCAUUAA 3'
                  | | | | | | | | | | | | | |
hsa-miR-155   3' GGGGAUAGUGCUAAUCGUAAUU 5'

b

Figure 4 SOCS1 expression is reduced in UC-derived IMF. (**a**) Schematic representation of putative miR-155-binding site in the 3′-untranslated region (3′-UTR) of SOCS1. (**b**) Protein levels of SOCS1 were determined by western blot analysis control- and UC-derived IMF. Densitometric quantification performed using β-actin as internal control ($n = 3$). *$P < 0.05$ vs control IMF.

this miRNA deregulation in colonic mucosa of UC patients has been recently underscored in the study by Singh et al.[35] showing that miR-155 deficit protects mice from experimental colitis.

IMF are plastic cells sensing and reacting to a variety of stress to preserve intestinal mucosa homeostasis.[3] However, in response to persistent inflammatory insults like in IBD, IMF acquire an activated phenotype, proliferate, cause an unnecessary remodeling of the extracellular matrix and produce an excess of soluble mediators, such as inflammatory cytokines, TGF-β1 and Wnts ligands, which profoundly influence neighboring epithelial, mesenchymal and immune cells. Compelling evidence indicates that miR-155 regulates several cellular functions involved in inflammatory processes, such as survival, growth and chemosensitivity. Therefore, deregulated miR-155 expression can profoundly impact cellular phenotype and functions.[12,13] In line with a previous report for the mono-cyte–macrophage lineage,[33] pro-inflammatory mediators such as TNF-α and LPS positively regulated miR-155 expression in IMF. Indeed, in macrophages TNF-α upregulates miR-155 that contributes to increased miR-155 production in an autocrine loop.[36] All together these data support the view that the persistently increased mucosal levels of TNF-α in UC patient[37] can drive miR-155 overexpression in IMF, influencing their phenotype. TGF-β1 failed to directly regulate miR-155 in
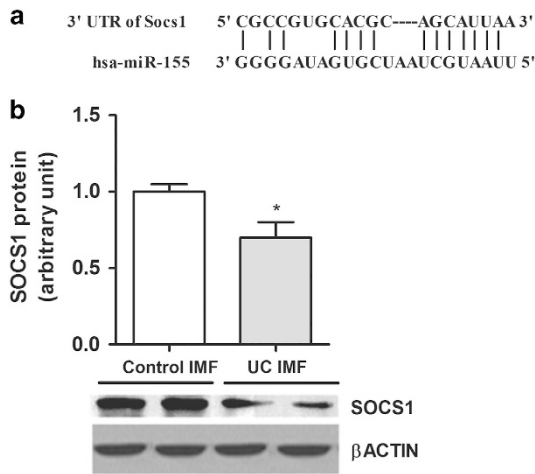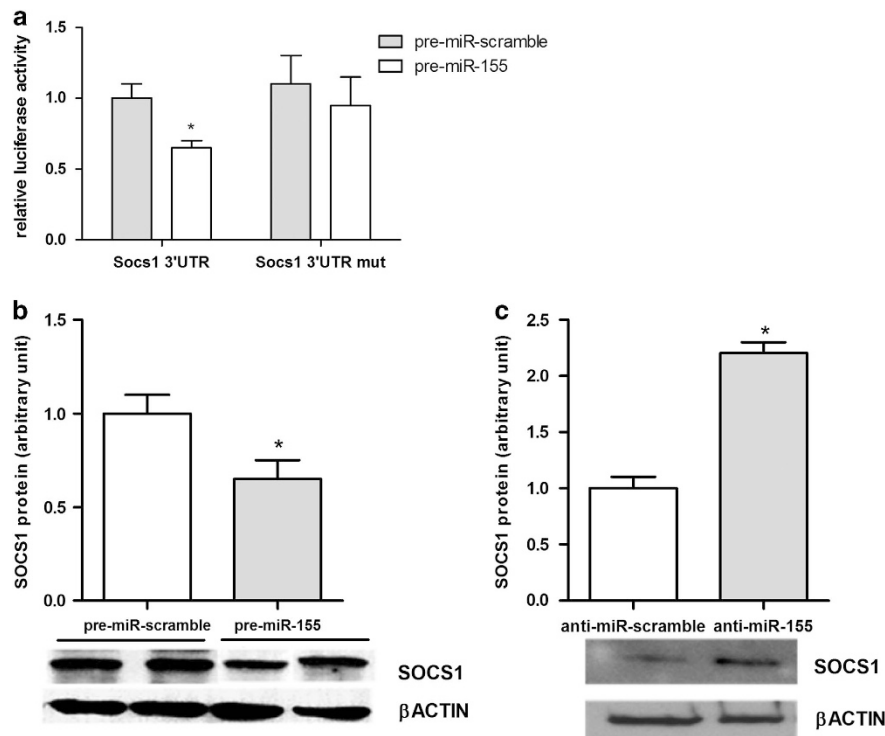
Figure 5 MiR-155 directly targets SOCS1 in IMF. (**a**) Luciferase activity in HEK 293A cells co-transfected with each reporter plasmid (pmir-GLO-Socs1 3′-UTR or pmir-GLO-Socs1 3′-UTR mut) and pre-miR-155 or pre-miR-scramble. *$P < 0.05$ vs pre-miRNA scramble. (**b**) Western blotting analysis of SOCS1 expression in control-derived IMF following pre-miRNA-155 transfection. Densitometric quantification performed using β-actin as internal control ($n = 3$). *$P < 0.05$ vs pre-miRNA scramble. (**c**) Western blot analysis of SOCS1 expression in UC-derived IMF following transfection of anti-miRNA-155 or scramble oligonucleotide as control ($n = 3$). *$P < 0.05$ vs anti-miRNA scramble control.
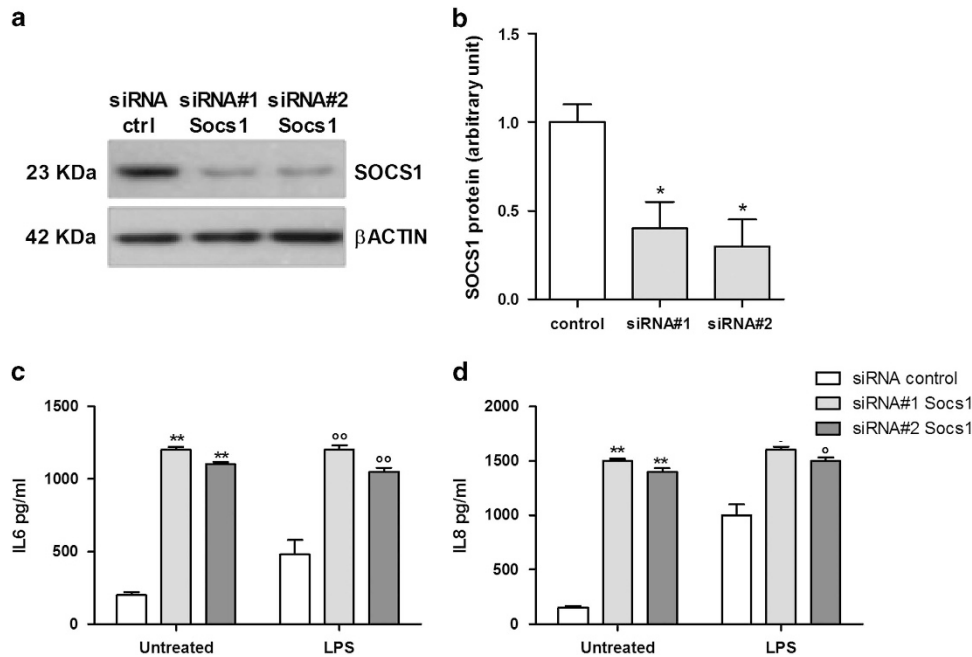
Figure 6 SOCS1 regulates inflammatory cytokines in IMF (**a**) Validation of SOCS1 silencing by western blot analysis of SOCS1 expression in control-derived IMF transfected with SOCS1 siRNA no. 1 and siRNA no. 2 or scramble control. (**b**) Densitometric quantification performed using β-actin as internal control ($n = 3$). *$P < 0.05$ vs scramble control. (**c**) IL-6 and (**d**) IL-8 release determined by ELISA in control-derived IMF supernatants following SOCS1 knockdown ($n = 3$). **$P < 0.01$ vs control, °°$P < 0.01$ and °$P < 0.05$ vs LPS alone.

IMF in contrast to the direct upregulation observed in lymphoma cell lines[38] and mammary gland adenocarcinoma cells.[39] Thus, taking into account the growth-inhibitory effects of TGF-β1, a miR-155-mediated mechanism to escape TGF-β's growth-inhibitory effects may be present in transformed cells but not in IMF isolated from non-cancerous tissues. Intriguingly, in several immune cells of IBD patients, the resistance to TGF-β1 signaling is due to enhanced activity of the inhibitory Smad7[40] as opposite to somatic mutations in SMADs or TGF-β receptors reported in malignant cells.[41]

On the basis of the fact that miR-155 induced inflammatory cytokine production in IMF, validation of the target genes was focused on putative candidates known to be associated with control of inflammatory processes, taking into account that miR-155 targets are estimated to be over 900.[42] However, only a minority of *in silico* predicted targets have been found to be responsive to miRNA control upon experimental validation.[42] Recent studies support a positive relationship between miR-155 upregulation and molecular events associated to inflammatory processes such as activation of nuclear factor kB.[43] Furthermore, Min *et al.*[16] recently identified FOXO3 as target of miR-155 in HT-29 cells where it contributes to the upregulation of inflammatory cytokines such as IL-8. Here, we identified SOCS1 as a direct target of miR-155, confirming recent functional studies in T-regulatory, Th17 and effector CD8 T lymphocytes, breast cancer cells, microglia and macrophages.[44–47] SOCS1 is a potent molecular switch that, tuning key signaling pathways such as the JAK kinases and activated cytokine-receptor complexes, regulates the development of a variety of cell populations, inflammatory processes

and immune responses.[48] Indeed, SOCS1-deficient mice, who are hypersensitive to TLR ligands, show a deregulated cytokine production that disturbs the activation of immune cells and triggers the development of systemic autoimmunity.[49] Interestingly, SOCS1 expression has been described in the small and large intestines of humans and rodents[50] and SOCS1 knockdown in mice deficient for T-cell receptor α markedly enhanced colitis severity, suggesting that this protein has a critical role in maintaining mucosal homeostasis.[28] Previous studies have suggested that SOCS1 is inactivated in human IBD contributing to deregulated mucosa inflammation, although the cellular populations involved were not identified.[27] Here, we identify IMF as a reduced source of mucosal SOCS1 and demonstrate that inadequate SOCS1 levels contribute to the inflammatory phenotype of these cells.

In summary, to our knowledge this study represents the first comprehensive demonstration that the expression of a specific miRNA has a key role in supporting the inflammatory phenotype of IMF in UC patients, a relevant pathophysiological process in IBD. Taken together, our data provide novel insight into the peculiar role of miR-155 in intestinal mucosa during chronic inflammatory disorders and identify one of its specific target genes in IMF. Thus, inhibiting miR-155 may represent an interesting and innovative therapeutic target to prevent the deregulated cytokine release from IMF that contributes to fueling the inflammation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

1 Geremia A, Biancheri P, Allan P, Corazza GR, Di Sabatino A. Innate and adaptive immunity in inflammatory bowel disease. *Autoimmun Rev* 2014; **13**: 3–10.

2 Corridoni D, Arseneau KO, Cominelli F. Inflammatory bowel disease. *Immunol Lett* 2014; **161**: 231–235.

3 Owens BM, Simmons A. Intestinal stromal cells in mucosal immunity and homeostasis. *Mucosal Immunol* 2013; **6**: 225–234.

4 Naylor AJ, Filer A, Buckley CD. The role of stromal cells in the persistence of chronic inflammation. *Clin Exp Immunol* 2013; **171**: 30–35.

5 Fabian MR, Sonenberg N. The mechanics of miRNA-mediated gene silencing: a look under the hood of miRISC. *Nat Struct Mol Biol* 2012; **19**: 586–593.

6 Avraham R, Yarden Y. Regulation of signalling by microRNAs. *Biochem Soc Trans* 2012; **40**: 26–30.

7 Miska EA. How microRNAs control cell division, differentiation and death. *Curr Opin Genet Dev* 2005; **15**: 563–568.

8 Esteller M. Non-coding RNAs in human disease. *Nat Rev Genet* 2011; **12**: 861–874.

9 Coskun M, Bjerrum JT, Seidelin JB, Nielsen OH. MicroRNAs in inflammatory bowel disease - pathogenesis, diagnostics and therapeutics. *World J Gastroenterol* 2012; **18**: 4629–4634.

10 Iborra M, Bernuzzi F, Invernizzi P, Danese S. MicroRNAs in autoimmunity and inflammatory bowel disease: crucial regulators in immune response. *Autoimmun Rev* 2012; **11**: 305–314.

11 Pekow JR, Kwon JH. MicroRNAs in inflammatory bowel disease. *Inflamm Bowel Dis* 2012; **18**: 187–193.

12 Vigorito E, Kohlhaas S, Lu D, Leyland R. miR-155: an ancient regulator of the immune system. *Immunol Rev* 2013; **253**: 146–157.

13 Elton TS, Selemon H, Elton SM, Parinandi NL. Regulation of the MIR155 host gene in physiological and pathological processes. *Gene* 2013; **532**: 1–12.

14 Takagi T, Naito Y, Mizushima K, Hirata I, Yagi N, Tomatsuri N *et al.* Increased expression of microRNA in the inflamed colonic mucosa of patients with active ulcerative colitis. *J Gastroenterol Hepatol* 2010; **25** (Suppl 1): S129–S133.

15 Wu F, Zikusoka M, Trindade A, Dassopoulos T, Harris ML, Bayless TM *et al.* MicroRNAs are differentially expressed in ulcerative colitis and alter expression of macrophage inflammatory peptide-2 alpha. *Gastroenterology* 2008; **135**: 1624–1635.

16 Min M, Peng L, Yang Y, Guo M, Wang W, Sun G. MicroRNA-155 is involved in the pathogenesis of ulcerative colitis by targeting FOXO3a. *Inflamm Bowel Dis* 2014; **20**: 652–659.

17 Fasseu M, Treton X, Guichard C, Pedruzzi E, Cazals-Hatem D, Richard C *et al.* Identification of restricted subsets of mature microRNA abnormally expressed in inactive colonic mucosa of patients with inflammatory bowel disease. *PLoS ONE* 2010; **5**: e13160.

18 Osada H, Ohkusa T, Yokoyama T, Shibuya T, Sakamoto N, Beppu K *et al.* Comparison of several activity indices for the evaluation of endoscopic activity in UC: inter- and intraobserver consistency. *Inflamm Bowel Dis* 2010; **16**: 192–197.

19 Sostegni R, Daperno M, Scaglione N, Lavagna A, Rocca R, Pera A. Review article: Crohn's disease: monitoring disease activity. *Aliment Pharmacol Ther* 2003; **17**: 11–17.

20 McKaig BC, Makh SS, Hawkey CJ, Podolsky DK, Mahida YR. Normal human colonic subepithelial myofibroblasts enhance epithelial migration (restitution) via TGF-β3. *Am J Physiol* 1999; **276**: 1087–1093.

21 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 2001; **25**: 402–408.

22 Di Sabatino A, Jackson CL, Pickard KM, Buckley M, Rovedatti L, Leakey NA *et al.* Transforming growth factor beta signalling and matrix metalloproteinases in the mucosa overlying Crohn's disease strictures. *Gut* 2009; **58**: 777–789.

23 Brenmoehl J, Miller SN, Hofmann C, Vogl D, Falk W, Schölmerich J *et al.* Transforming growth factor-beta 1 induces intestinal myofibroblast differentiation and modulates their migration. *World J Gastroenterol* 2009; **15**: 1431–1442.

24 McKaig BC, Hughes K, Tighe PJ, Mahida YR. Differential expression of TGFbeta isoforms by normal and inflammatory bowel disease intestinal myofibroblasts. *Am J Physiol Cell Physiol* 2002; **282**: C172–C182.

25 Bajaj-Elliott M, Breese E, Poulsom R, Fairclough PD, MacDonald TT. Keratinocyte growth factor in inflammatory bowel disease. Increased mRNA transcripts in ulcerative colitis compared with Crohn's disease in biopsies and isolated mucosal myofibroblasts. *Am J Pathol* 1997; **151**: 1469–1476.

26 McKaig BC, McWilliams D, Watson SA, Mahida YR. Expression and regulation of tissue inhibitor of metalloproteinase-1 and matrix metalloproteinases by intestinal myofi-broblasts in inflammatory bowel disease. *Am J Pathol* 2003; **162**: 1355–1360.

27 Horino J, Fujimoto M, Terabe F, Serada S, Takahashi T, Soma Y *et al.* Suppressor of cytokine signaling-1 ameliorates dextran sulfate sodium-induced colitis in mice. *Int Immunol* 2008; **20**: 753–762.

28 Chinen T, Kobayashi T, Ogata H, Takaesu G, Takaki H, Hashimoto M *et al.* Suppressor of cytokine signaling-1 regulates inflammatory bowel disease in which both IFNgamma and IL-4 are involved. *Gastroenterology* 2006; **130**: 373–388.

29 Danese S. Immune and nonimmune components orchestrate the pathogenesis of inflammatory bowel disease. *Am J Physiol Gastrointest Liver Physiol* 2011; **300**: G716–G722.

30 O'Connell RM, Rao DS, Chaudhuri AA, Baltimore D. Physiological and pathological roles for microRNAs in the immune system. *Nat Rev Immunol* 2010; **10**: 111–122.

31 Chen WX, Ren LH, Shi RH. Implication of miRNAs for inflammatory bowel disease treatment: Systematic review. *World J Gastrointest Pathophysiol* 2014; **5**: 63–70.

32 Iborra M, Bernuzzi F, Correale C, Vetrano S, Fiorino G, Beltrán B *et al.* Identification of serum and tissue micro-RNA expression profiles in different stages of inflammatory bowel disease. *Clin Exp Immunol* 2013; **173**: 250–258.

33 Tili E, Michaille JJ, Cimino A, Costinean S, Dumitru CD, Adair B *et al.* Modulation of miR-155 and miR-125b levels following lipopolysaccharide/ TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. *J Immunol* 2007; **179**: 5082–5089.

34 O'Connell RM, Kahn D, Gibson WS, Round JL, Scholz RL, Chaudhuri AA *et al.* MicroRNA-155 promotes autoimmune inflammation by enhancing inflammatory T cell development. *Immunity* 2010; **33**: 607–619.

35 Singh UP, Murphy AE, Enos RT, Shamran HA, Singh NP, Guan H *et al.* miR-155 deficiency protects mice from experimental colitis by reducing Th1/Th17 responses. *Immunology* 2014; **143**: 478–489.

36 Bala S, Marcos M, Kodys K, Csak T, Catalano D, Mandrekar P *et al.* Up-regulation of microRNA-155 in macrophages contributes to increased tumor necrosis factor α production via increased mRNA half-life in alcoholic liver disease. *J Biol Chem* 2011; **286**: 1436–1444.

37 Matsuda R, Koide T, Tokoro C, Yamamoto T, Godai T, Morohashi T *et al.* Quantitive cytokine mRNA expression profiles in the colonic mucosa of patients with steroid naïve ulcerative colitis during active and quiescent disease. *Inflamm Bowel Dis* 2009; **15**: 328–334.

38 Rai D, Kim SW, McKeller MR, Dahia PL, Aguiar RC. Targeting of SMAD5 links microRNA-155 to the TGF-beta pathway and lymphomagenesis. *Proc Natl Acad Sci USA* 2010; **107**: 3111–3116.

39 Kong W, Yang H, He L, Zhao JJ, Coppola D, Dalton WS *et al.* MicroRNA-155 is regulated by the transforming growth factor beta/Smad

pathway and contributes to epithelial cell plasticity by targeting RhoA. *Mol Cell Biol* 2008; **28**: 6773–6784.

40 Monteleone G, Kumberova A, Croft NM, McKenzie C, Steer HW, MacDonald TT. Blocking Smad7 restores TGF-β1 signaling in chronic inflammatory bowel disease. *J Clin Invest* 2001; **108**: 601–609.

41 Wang J, Yang L, Yang J, Kuropatwinski K, Wang W, Liu XQ *et al.* Transforming growth factor beta induces apoptosis through repressing the phosphoinositide 3-kinase/AKT/survivin pathway in colon cancer cells. *Cancer Res* 2008; **68**: 3152–3160.

42 Faraoni I, Antonetti FR, Cardone J, Bonmassar E. miR-155 gene: a typical multifunctional microRNA. *Biochim Biophys Acta* 2009; **1792**: 497–505.

43 Wang B, Majumder S, Nuovo G, Kutay H, Volinia S, Patel T *et al.* Role of microRNA-155 at early stages of hepatocarcinogenesis induced by choline-deficient and amino acid-defined diet in C57BL/6 mice. *Hepatology* 2009; **50**: 1152–1161.

44 Yao R, Ma YL, Liang W, Li HH, Ma ZJ, Yu X *et al.* MicroRNA-155 modulates Treg and Th17 cells differentiation and Th17 cell function by targeting SOCS1. *PLoS ONE* 2012; **7**: e46082.

45 Dudda JC, Salaun B, Ji Y, Palmer DC, Monnot GC, Merck E. MicroRNA-155 is required for effector CD8+ T cell responses to virus infection and cancer. *Immunity* 2013; **38**: 742–753.

46 Wang P, Hou J, Lin L, Wang C, Liu X, Li D *et al.* Inducible microRNA-155 feedback promotes type I IFN signaling in antiviral innate immunity by targeting suppressor of cytokine signaling 1. *J Immunol* 2010; **185**: 6226–6233.

47 Cardoso AL, Guedes JR, Pereira de Almeida L, Pedroso de Lima MC. miR-155 modulates microglia-mediated immune response by down-regulating SOCS-1 and promoting cytokine and nitric oxide production. *Immunology* 2012; **135**: 73–88.

48 Linossi EM, Babon JJ, Hilton DJ, Nicholson SE. Suppression of cytokine signaling: the SOCS perspective. *Cytokine Growth Factor Rev* 2013; **24**: 241–248.

49 Yoshimura A. Regulation of Cytokine Signaling by the SOCS and Spred Family Proteins. *Keio J Med* 2009; **58**: 73–83.

50 Suzuki A, Hanada T, Mitsuyama K, Yoshida T, Kamizono S, Hoshino T *et al.* CIS3/SOCS3/SSI3 plays a negative regulatory role in STAT3 activation and intestinal inflammation. *J Exp Med* 2001; **193**: 471–481.