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RESEARCH PAPER

AM3, a natural glycoconjugate, induces the functional maturation of human dendritic cells

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Background and purpose: Dendritic cells (DCs) are dedicated antigen-presenting cells able to initiate specific immune responses and their maturation is critical for the induction of antigen-specific T-lymphocyte responses. Here, we have investigated the effects of Inmunoferon-active principle (AM3), the active agent of a commercial immunomodulatory drug, on human monocyte-derived DCs (MDDCs).
Experimental approach: MDDCs derived from healthy and hepatitis C virus (HCV)-infected patients were stimulated with AM3. We analysed the expression of cell surface proteins by flow cytometry, that of cytokine production by ELISA, and the expression of chemokines and chemokine receptors by RNase protection assays. T-lymphocyte proliferation was assessed in mixed lymphocyte reactions, protein expression by western blot and luciferase-based reporter methods, and Toll-like receptor (TLR)-blocking antibodies were employed to analyse TLR activity.
Key results: In MDDCs, AM3 induced or enhanced expression of CD54, CD83, CD86, HLA-DR, chemokines and chemokine receptors, interleukin (IL)-12p70 and IL-10. Furthermore, AM3 stimulated MDDCs to increase proliferation of allogenic T cells. AM3 triggered nuclear translocation of NF-κB and phosphorylation of p38 mitogen-activated protein kinase. AM3 promoted NF-κB activation in a TLR-4-dependent manner, and blocking TLR-4 activity attenuated the enhanced expression of CD80, CD83 and CD86 induced by AM3. AM3 enhanced the expression of maturation-associated markers in MDDCs from HCV-infected patients and increased the proliferation of T lymphocytes induced by these MDDCs.
Conclusions and implications: These results underline the effects of AM3 in promoting maturation of MDDCs and suggest that AM3 might be useful in regulating immune responses in pathophysiological situations requiring DC maturation.
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Keywords: dendritic cells; immunomodulation; glycoconjugate; hepatitis C virus

Abbreviations: AM3, Inmunoferon-active principle; DC, dendritic cell; LPS, lipopolysaccharide; MDDC, monocyte-derived dendritic cell; TLR, Toll-like receptor; MAPK, mitogen-activated protein kinase; MLR, mixed lymphocyte reaction

Introduction

Dendritic cells (DCs) are the most potent antigen-presenting cells in the immune system. They exist in two functionally and phenotypically distinct states, termed mature and immature. Immature DCs are located in many different tissues and organs, and they act as host-defence cells, capturing foreign antigens with high efficiency. When confronted with a pathogen challenge or other perceived 'danger', DCs migrate to the peripheral lymphoid organs where they undergo profound changes in phenotype and function, a process referred to as DC maturation. Different stimuli such as pro-inflammatory cytokines (for example, tumour necrosis factor-α and interleukin-1 (IL-1)) or bacterial products such as lipopolysaccharide (LPS) can induce DC maturation *in vivo* and *in vitro*. Although this process is accompanied by a decrease in their capacity for endocytosis and phagocytosis, as well as antigen uptake and processing, it does result in increased antigen presentation (Cella *et al.*, 1997; Banchereau *et al.*, 2000). After maturation, DCs produce cytokines such as IL-1, IL-10 and IL-12, which are

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essential for the polarization of the T-cell response towards Th1 or Th2 (de Saint-Vis *et al*., 1998). Also, they produce chemokines such as CCL2, CCL3, CCL4 and CXCL8, which favour lymphocyte recruitment and activation (Moser *et al*., 2004). In addition, mature DCs express increased levels of surface antigens involved in T-cell activation, such as the co-stimulatory molecules CD80 and CD86 and major histocompatibility complex-I (MHC-I) and MHC-II molecules. In conjunction, these events result in enhanced antigen presentation and a greater capacity to promote T-cell proliferation (Cella *et al*., 1997; Banchereau *et al*., 2000).

Immunoferon is an orally administered immunoregulatory glycoconjugate that consists of a phosphorylated glucomannan polysaccharide from *Candida utilis* and a storage protein from non-germinated seeds of *Ricinus communis* (*Euphorbiacea*; Varela *et al*., 2002). Its active ingredient is a 5:1 (w/w) mixture of polysaccharide and protein, called Inmunoferon-active principle (AM3) (Brieva *et al*., 2001). The protein component of AM3 is the naturally occurring RicC3, a member of the 2S albumin family (Varela *et al*., 2002). In turn, 2S albumins belong to the prolamine superfamily, some members of which are food allergens. RicC3 (12.0 kDa) consists of a small and a large subunit that form a heterodimer linked by very stable disulphide bridges resistant to denaturation, acidic pH and proteolytic cleavage (Pantoja-Uceda *et al*., 2002). NMR structures of recombinant RicC3 indicate the presence of a bundle of five α-helixes folded in a right-handed superhelix (Pantoja-Uceda *et al*., 2003). The polysaccharide component of AM3 is a phosphoglucomannan (GLPH-1; approximately 15 kDa), which contains a repeated polysaccharide subunit (10–40 repeats), with 1–6 and 1–2 linkages between and within the mannose and glucose residues at a ratio of 12:1 mannose:glucose (Bermejo J, personal communication).

AM3 modulates the regulatory and effector functions of the immune system, acting on peripheral blood mononuclear cells *in vitro* and *in vivo* (Majano *et al*., 2004), as well as enhancing lymphocyte proliferation, IL-2 production and the cytotoxicity of natural killer cells (Rojo *et al*., 1986). In animal models, the capacity of AM3 to potentiate natural (innate) and specific immunity is related to the induction of endogenous IL-12 and interferon (IFN) production (Villarrubia *et al*., 1997) and the partial inhibition of tumour necrosis factor-α production (Brieva *et al*., 2001) as well as inducible NOS expression (Majano *et al*., 2005). The ability of AM3 to improve immune activity clinically has been confirmed by the restoration of cutaneous delayed-type hypersensitivity reactions in children with asthma (Sánchez Palacios *et al*., 1992). Similarly, AM3 reduces recurrent aphthous ulcers in oral stomatitis (Camacho *et al*., 1991) but improves viral clearance in chronic hepatitis B carriers (Villarrubia *et al*., 1992). In patients with chronic obstructive pulmonary disease, AM3 is able to normalize the deficient effector function of natural killer, phagocytotic cells (Prieto *et al*., 2001) and T lymphocytes (Reyes *et al*., 2006). Interestingly, AM3 has also been shown to be an effective adjuvant in hepatitis B vaccination of healthy people who previously failed to develop HbsAg (hepatitis B surface antigen) titres $>10\,\mathrm{IU\,mL^{-1}}$ in response to the recombinant hepatitis B vaccine (Sanchez *et al*., 1995), as well as in haemodialysis patients who were non-responders to hepatitis B (Perez-García *et al*., 2002).

Hepatitis C virus (HCV) is a major cause of chronic hepatitis, cirrhosis and hepatocarcinoma (Poynard *et al*., 2003). Clearance of HCV can occur during acute infection and is associated with a vigorous, long-lasting cellular immune response against multiple HCV epitopes. In approximately 80% of patients, insufficient elimination of the HCV during acute infection results in chronic disease development due to weak virus-specific immune response (Gremion and Cerny, 2005). Inefficient induction of T-cell-mediated responses or dysfunction of DCs has also been proposed to contribute to the causes of this impairment (Pachiadakis *et al*., 2005).

Although AM3 is administered to induce nonspecific activation of the immune system and to prevent recurrent infections, the mechanism by which AM3 acts remain largely unexplored. In this study, we have demonstrated that AM3 induces functional maturation of monocyte-derived DCs (MDDCs) derived from healthy donors and patients with chronic HCV infection, which may explain some of the effects associated with AM3 administration.

Materials and methods

Generation and immunophenotyping of MDDCs

Peripheral blood mononuclear cells were purified from healthy donors by Ficoll density centrifugation (Histopaque-1077; Sigma Co., St Louis, MO, USA), and $CD14^+$ cells were purified by positive selection using anti-$CD14^+$ microbeads and the MiniMACS system following the manufacturer's instructions (Miltenyi Biotec, Auburn, CA, USA). $CD14^+$ cells were cultured in six-well plates (Costar, Cambridge, MA, USA) at 1×10^6 cells per mL RPMI 1640 (Life Technologies, Merelbeke, Belgium) containing 10% foetal calf serum and $20\,\mu\mathrm{g\,mL^{-1}}$ gentamicin, supplemented with granulocyte–macrophage colony-stimulating factor ($1000\,\mathrm{U\,mL^{-1}}$) and IL-4 ($1000\,\mathrm{U\,mL^{-1}}$; Preprotech, Rocky Hill, NJ, USA). Fresh medium containing granulocyte–macrophage colony-stimulating factor and IL-4 was added every 2–3 days and human MDDCs were routinely used on days 5–6 of culture. All the experiments were carried out in RPMI containing 10% foetal calf serum except those to determine mitogen-activated protein kinase (MAPK) levels where immature MMDCs were cultured with medium containing 0.5% foetal calf serum 16 h before treatment. Cell viability was estimated with propidium iodide or Trypan Blue to rule out the possibility that some of the effects of AM3 were due to toxicity.

Antibodies against the following proteins were employed in this study: HLA-DR (DR); CD54/ICAM-1 (Hu5/3); ICAM-3 (TP1/24); kindly provided by Dr Francisco Sanchez-Madrid (Hospital Universitario de la Princesa, Madrid, Spain) and fluorescein isothiocianate (FITC)-conjugated antibodies against CD80, CD86 and CD83 purchased from Caltag Laboratories (Burlingame, CA, USA). Immunostaining of unstimulated or stimulated MDDCs was performed by incubating 10^5 cells with the aforementioned antibodies or their isotype-matched controls for 20 min at 4 °C. The cells

were then rinsed with ice-chilled phosphate-buffered saline (PBS) and, where necessary, a secondary FITC-conjugated goat anti-rabbit antibody (Dako, Glostrup, Denmark) was used. Finally, the fluorescence intensity of the cells was measured using a FACSCalibur flow cytometer (BD Bioscience, Franklin Lakes, NJ, USA).

FITC-dextran uptake by MDDCs
To measure particle uptake by MDDCs, 5×10^4 cells were resuspended in 100 μL of PBS containing 1% human serum and incubated with FITC-dextran (0.1 mg mL^{-1}, Sigma Co.) at either 37 or 4 °C for 30 min. The process was stopped by adding 2 mL ice-cold PBS containing 1% human serum and the cells were then washed three times with ice-cold PBS before they were analysed by flow cytometry.

Measurement of cytokine levels
The IL-12 p70 and IL-10 in the culture supernatants from MDDCs were assayed with enzyme-linked immunosorbent assays (ELISA kits, R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. IFN-γ and IL-4 were determined in supernatants of MDDCs/T lymphocytes after 5 days co-culture using standard ELISA immunoassays according to the manufacturer's protocols (Pierce Endogen, Rockford, IL, USA).

Allogeneic T-lymphocyte proliferation induced by MDDCs
Peripheral blood mononuclear cells were obtained from healthy adults as described above. Allogeneic CD3$^+$ T lymphocytes were isolated by negative immunomagnetic selection using a Pan T cell Isolation Kit II human (Miltenyi Biotec) according to the manufacturer's instructions. In T-lymphocyte proliferation experiments, 2×10^5 T cells were stimulated in a 96-well plate with 0.1, 0.5, 2.5 or 10×10^3 irradiated (1.5 Gy min^{-1} for 10 min) allogeneic MDDCs that were matured under different culture conditions. After a 5-day incubation period, tritiated thymidine was added (0.037 MBq per well) during the last 16 h of co-culture and the incorporation of thymidine was determined to assess the level of T-cell proliferation.

Determination of NF-κB activity
Monocyte-derived DCs were plated on gelatin-coated coverslips and they were allowed to settle for 30 min before they were treated with LPS (0.1 μg mL^{-1}) or AM3 (1 μg mL^{-1}) for different times (15 min to 4 h). The subcellular localization of NF-κB was analysed by immunofluororescence with a specific polyclonal antiserum against RelA/p65 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Briefly, cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, permeabilized for 5 min in PBS containing 0.1% Triton X-100, blocked for 30 min at 37 °C with BSA (Boehringer Mannheim, GmbH, Mannheim, Germany) and incubated for 1 h with an 1:1000 dilution of the antiserum. The cells were then washed with PBS and labelled with a Cy3-conjugated rabbit anti-goat antibody (Jackson Immuno Research Laboratories, Inc, West Grove, PA, USA). The coverslips were mounted in fluorescent mounting medium (Dako) and representative fields were photographed on a Nikkon Eclipse E-800 microscope (Nikkon, Melville, NJ, USA).

MAPK and IkBα expression
Protein levels were measured in unstimulated MDDCs or MDDCs stimulated with LPS or AM3 for 5 min to 24 h by western blotting using specific polyclonal antisera against ERK 1/2, p38, phospho-ERK 1/2, phospho-p38 (Cell Signaling, Beverly, MA, USA) and IkBα (Santa Cruz Biotechnology), as described previously (Puig-Kröger *et al*., 2001).

Chemokine and chemokine receptor mRNA expression
Dendritic cells were stimulated for 12 h with LPS or AM3, and total RNA was extracted from cultured cells using the ULTRASPEC RNA isolation system (Biotecx Laboratories, Houston, TX, USA). The expression of human chemokine and chemokine receptor mRNAs was determined by RNase protection assays using the multiprobe template set hCK5 (containing DNA templates for XCL1, CCL5, CXCL10, CCL3, CCL4, CCL2, CXCL8, CCL1), hCR5 (CCR1, CCR3, CCR4, CCR5, CCR8, CCR2a + b, CCR2a, CCR2b) and hCR6 (CXCR1, CXCR2, CXCR3, CXCR4, CXCR5/BLR-1, CCR7/BLR-2, V28/CX3CR1) (Pharmingen, San Diego, CA, USA) following the manufacturer's protocols. Relative mRNA levels were quantified by PhosphorImager scanning using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA).

Determination of interactions between AM3 and TLR 2 or TLR 4
HEK293-Toll-like receptor (TLR)4 and HEK293-TLR2 cells (kindly provided by Dr Douglas T Golenbock, University of Massachussetts, Worcester, MA, USA) that have been stably transfected with human TLR4 and TLR2, respectively, were transiently transfected with 1 μg of the κB-Luc reporter vector containing three H-2K^b gene NF-κB motifs upstream of the luciferase reporter gene (Yano *et al*., 1987). The cells were transfected using Superfect (Quiagen, Valencia, CA, USA) according to the manufacturer's recommendations, and after 24 h they were trypsinized and plated in 96-well plates (10^3 cells per well) for 12 h. Following exposure to different concentrations of AM3 for 6 h, the cells were harvested and lysed and the luciferase activity was determined using the Luciferase Assay System Kit (Promega, Valencia, CA, USA). As positive controls, HEK293-TLR4 cells were stimulated with LPS and HEK293-TLR2 cells with Pam3Cys (Invivogen, San Diego, CA, USA). In additional experiments, MDDCs were incubated with medium containing either a TLR4-blocking (clone HTA 125; Invivogen. San Diego, CA, USA) or isotype matched antibody (both at 1.5 μg mL^{-1}) for 30 min before AM3 or LPS treatment.

Chronically infected patients
Eight patients with chronic hepatitis C who had been referred to the Liver Unit of the Hospital Universitario de

Table 1 Characteristics of HCV-infected patients

Patient	*Age*	*Ferritin level* ($ng\,mL^{-1}$)	*Viral load* ($U\,mL^{-1}) \times 10^5$	*GPT/GOT* ($U\,L^{-1}$)	*Virus genotype*
1	56	1280	2.5	54/35	1b
2	50	898	49	81/71	1b
3	53	514	12	112/80	1
4	66	1266	3.4	129/81	1b
5	56	321	2.8	307/234	1b
6	48	508	5.4	58/40	1b
7	69	680	11	87/66	1b
8	50	460	16	112/78	1b
Mean	56	740.88	12.76		
s.d.	7.69	369.50	15.46		

GPT indicates alanine amino transferase.
GOT indicates aspartate amino transferase.
None of the patients had undergone antiviral treatment.

la Princesa (Madrid, Spain) were enrolled in this study, whose characteristics are shown in Table 1. Informed consent was obtained from each patient and the study protocol was approved by the Hospital's Ethical Committees for experiments involving humans. All patients were confirmed to be positive for HCV RNA (Cobas Amplicor Hepatitis C Monitor Test, v2.0 Roche Diagnostics, GmbH, Mannheim, Germany) and they were infected by HCV genotype 1 (INNO-LIPA HCV, Innogenetics, Gent, Belgium), whereas they were negative for other viral infections, including HBV and HIV. All patients had elevated baseline serum levels of iron and ferritin, and repeated phlebotomies of 400 mL were carried out until these abnormal levels reached the normal range before initiating combined antiviral therapy. Immunophenotyping and allogeneic T-lymphocyte proliferation assays were performed as above.

Reproducibility of the data between different donors and AM3 batches

Monocyte-derived DCs were generated from peripheral blood mononuclear cells isolated from healthy donors and at least three independent replicates were performed for each set of experiments. It is important to note that although the values showed slight variation between donors, the overall tendency remained unchanged. Two different batches of AM3 were used for the experiments reported with comparable results.

Data and statistical analysis

Results are given as mean ± s.d. Comparison between groups was carried out by using a mean paired *t*-test. The statistical program GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA, USA) was used.

Reagents

Lipopolysaccharide from *Escherichia coli* serotype (055:B5) was purchased from Sigma Chemical Co. (St Louis, MO, USA). AM3 was prepared according to the methods described in the patents P9900408 (Spain) and PCT/ES99/00338. Briefly, the phosphoglucomannan (GLPH-1 polysaccharide absorbed on a calcium phosphate matrix, 10/90 w/w) obtained from the cell wall of *Candida utilis* and the RicC3 protein from *Ricinus communis* seeds were combined at a ratio of 5:1 (w/w) polysaccharide/protein (Brieva *et al*., 2001). AM3 was assayed for bacterial endotoxin using the Pyrogent plus Test kit (Bio Whittaker, Rockland, ME, USA) that has a detection threshold of $0.0625\,IU\,mL^{-1}$. No endotoxin was detected at concentrations up to 1000-fold higher than those used in the experiments described here.

Results

AM3 induces human MDDC maturation

Immature DCs comparable to those found in non-lymphoid tissues can be generated by culturing human peripheral blood monocytes in medium supplemented with granulocyte–macrophage colony-stimulating factor and IL-4 (Sallusto and Lanzavecchia, 1994). To assess the effect of AM3 on MDDCs, we maintained human MDDCs in the presence or absence of AM3 for 24 h and then assessed the expression of a selected panel of DC markers, including the MHC class II molecule HLA-DR; CD86/B7-2, a co-stimulatory molecule required for T-cell activation; CD83, a specific marker of mature DCs and the adhesion molecules CD54/ICAM-1 and ICAM-3 (Figure 1). In initial experiments, we examined the effect of different concentrations of AM3 ranging from 0.1 to $10\,\mu g\,mL^{-1}$. Although $0.1\,\mu g\,mL^{-1}$ of AM3 promoted phenotypic changes in surface markers after stimulation of MDDCs, higher AM3 doses of 1 and $10\,\mu g\,mL^{-1}$ induced more pronounced modifications. However, stimulation with $10\,\mu g\,mL^{-1}$ of AM3 affected cell viability in some donors and, thus, we decided to use $1\,\mu g\,mL^{-1}$ of AM3 to stimulate MDDCs in most of the following experiments. AM3 induced a marked upregulation in the expression of all the markers tested (Figure 1). The phenotypic changes induced by AM3 were comparable to those elicited by LPS ($0.1\,\mu g\,mL^{-1}$), a positive control known to promote MDDC maturation (Figure 1), suggesting that AM3 induces MDDC maturation.

To further confirm that AM3 induced the maturation of MDDCs, we measured FITC-dextran uptake, which diminishes during DC maturation. Consistent with the phenotype changes in MDDCs, particle uptake also fell in AM3-stimulated MDDCs when compared with unstimulated MDDCs (Figure 2), further supporting the notion that AM3 promotes DC maturation.

AM3 enhances bioactive IL-12 and IL-10 production by MDDCs

To investigate whether the phenotypic switch induced by AM3 correlated with a change in cytokine expression, we analysed the expression of IL-10 and IL-12p70 whose expression is induced during MDDC maturation in response to LPS (Langenkamp *et al*., 2000). AM3 did indeed induce significant expression of IL-12 p70, although to a lesser extent than LPS (Figure 3). However, both AM3 and LPS induced strong expression of IL-10.

Enhancement of T-cell proliferation and activation by AM3-treated MDDCs

The increased expression of surface markers involved in antigen presentation to T cells and the increased IL-12

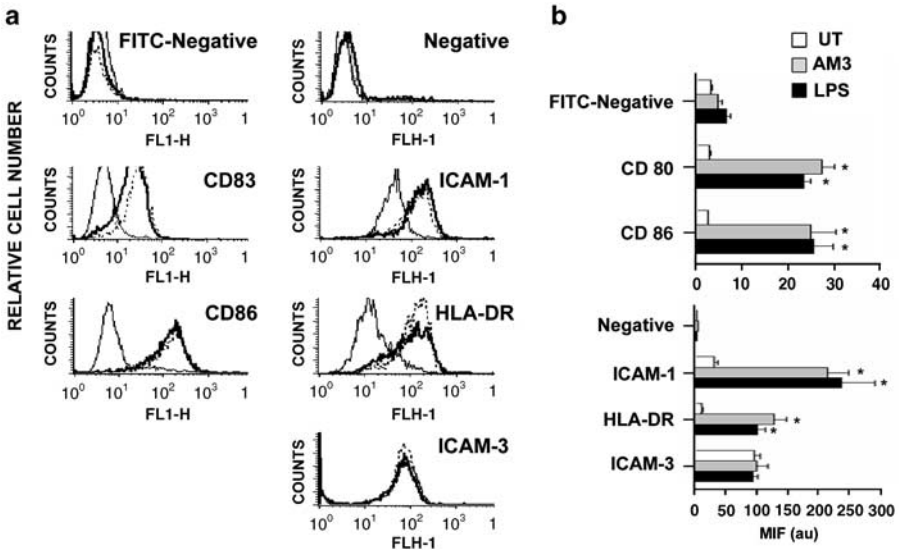

Figure 1 AM3 upregulates cell surface molecules on human MDDCs. (**a**) MDDCs were cultured for 16 h in the presence of AM3 (1 μg mL^{-1}, dotted line), LPS (0.1 μg mL^{-1}, bold line) or medium alone (thin line), and surface markers were analysed by flow cytometry as stated in Materials and methods. A representative experiment from one of the eight independent donors is shown. (**b**) Quantification of the experiments shown in (**a**). The data represent the mean intensity fluorescence (MIF) ± s.d. of eight independent donors. *$P<0.0001$ vs untreated cells (UT) MDDCs: paired *t*-test). LPS, lipopolysaccharide; MDDC, monocyte-derived dendritic cell.

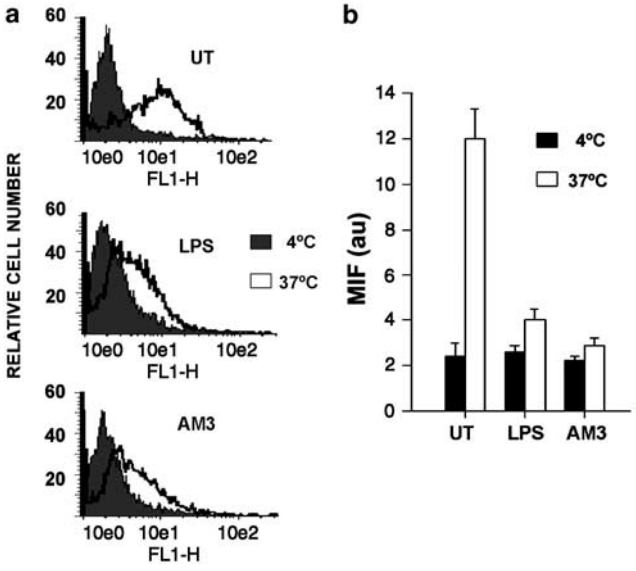

Figure 2 AM3 diminishes FITC-dextran uptake by MDDCs. (**a**) MDDCs were left untreated or stimulated with AM3 (1 μg mL^{-1}) or LPS (0.1 μg mL^{-1}) for 16 h and FITC-dextran uptake was measured by flow cytometry. The shading represents FITC-dextran uptake at 4 °C. One representative experiment is shown. (**b**) Quantification of the experiments shown in (**a**). UT, untreated cells. The data represents the mean intensity fluorescence (MIF) ± s.d. of three independent experiments. FITC, fluorescein isothiocianate; LPS, lipopolysaccharide; MDDC, monocyte-derived dendritic cell.

production observed in MDDCs exposed to AM3 suggested that this compound could activate T cells in allogenic T-cell responses. Mature DCs have the capacity to induce the proliferation of allogenic T cells more efficiently than immature DCs (Cella *et al*., 1997; Banchereau *et al*., 2000). Thus, we tested this possibility by exposing MDDCs to AM3 for 24 h and then incubating different numbers of AM3-treated DCs for 5 days with a constant number of purified allogeneic T cells. MDDCs activated by AM3 enhanced the proliferation of allogenic T cells to a similar extent as LPS-treated MDDCs (Figure 4a). In addition, AM3-treated MDDCs enhanced T-cell activation, as seen by the increase in IFN-γ secretion into the culture supernatant (Figure 4b). In contrast, little or no change in IL-4 production was observed upon exposure to AM3 or LPS alone (not shown).

AM3 upregulates chemokines and chemokine receptor expression

Upon stimulation, DCs produce the cytokines and chemokines that are involved in leukocyte recruitment (Sallusto *et al*., 1998, 1999). We tested the effect of AM3 on chemokine mRNA expression by DCs and found that in the presence of AM3 there was an increase in the expression of mRNA transcripts encoding for the chemokines CCL3, CCL4, CXCL8 and CCL2 (Figure 5a). In contrast, AM3 did not modify the expression of CCL5 (Figure 5a). AM3 also induced the expression of mRNA encoding for the chemokine receptors CXCR4 and CCR7, whereas the mRNA transcripts for CCR1 were reduced and those for CCR5 remained unaffected (Figure 5b).

AM3 induces NF-κB activation, IκB-α degradation and MAPK phosphorylation

Dendritic cells maturation is fully dependent on NF-κB activation, which ultimately determines most of the phenotypic and functional parameters associated with this process (Ardeshna *et al*., 2000; Neumann *et al*., 2000). To determine the molecular mechanism behind the AM3-induced maturation of MDDCs, we monitored its ability to trigger NF-κB translocation into the nucleus. As observed with LPS,

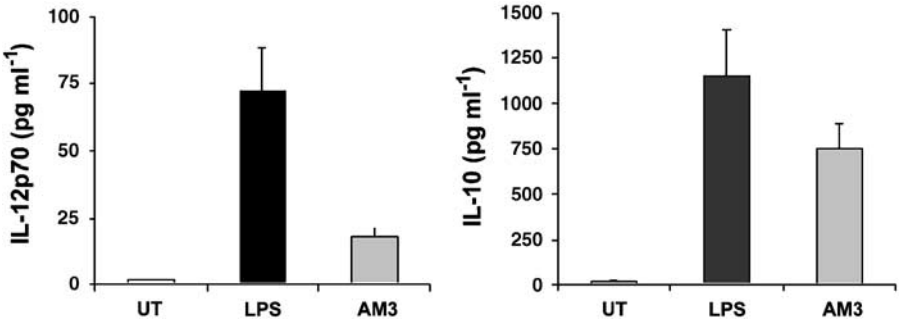

Figure 3 AM3 induces IL-12p70 and IL-10 production by human DCs. Human MDDCs were treated with AM3 (1 μg mL^{-1}) or LPS (0.1 μg mL^{-1}) for 24 h and then IL-12p70 (left) and IL-10 (right) production was analysed by ELISA. Experiments were performed in triplicate and the data represent the mean ± s.d. from four independent donors. DC, dendritic cell; IL, interleukin; LPS, lipopolysaccharide; MDDC, monocyte-derived DC.

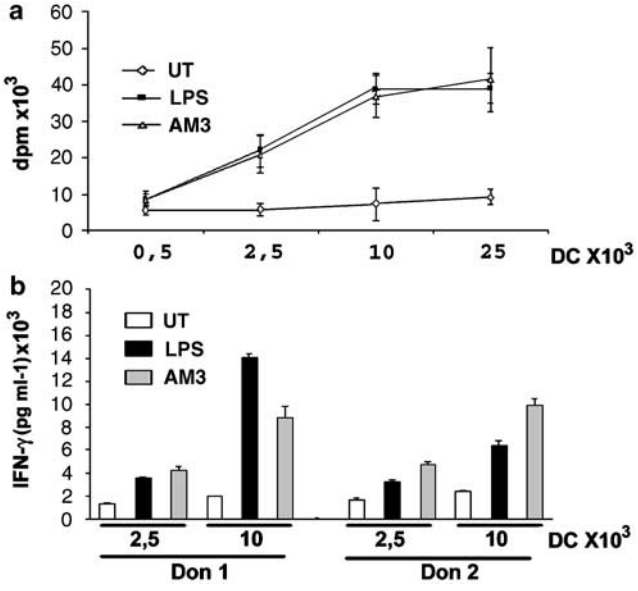

Figure 4 AM3 increases the proliferation of allogeneic T cells and IFN-γ production. (**a**). MDDCs were cultured in medium with or without AM3 (1 μg mL^{-1}) or LPS (0.1 μg mL^{-1}) for 24 h. An MLR was conducted for 5 days, as described in Materials and methods, and the background level of [^{3}H] TdR uptake was determined by measuring the reactions without stimulation. The values are the mean of triplicate ± s.d. The experiment was carried out with four independent donors and one representative experiment is shown. (**b**). ELISA analysis of IFN-γ in 6-day MLR supernatants (stimulator/responder ratio 1/80 and 1/20). The experiment was performed on five different donors and two representative donors are shown. The values are the mean of duplicate ± s.d. IFN, interferon; LPS, lipopolysaccharide; MDDC, monocyte-derived dendritic cell; MLR, mixed lymphocyte reaction.

exposing MDDCs to AM3 induced NF-κB (p65/RelA) nuclear translocation, as determined by immunocytochemistry (Figure 6a and data not shown). To further characterize the mechanism of action of AM3, MDDCs were treated with AM3 (1 μg mL^{-1}) or LPS (0.1 μg mL^{-1}) at different times and IκB-α levels were determined by western blotting. Exposure to AM3 induced a rapid and transient reduction in IκB-α that began to reverse after 2 h (Figure 6b). In contrast, the downregulation of IκB-α induced by LPS was not reversed until 24 h after treatment, suggesting that the effects of AM3 are less persistent than those of LPS.

The three major mammalian MAPKs signalling pathways, involving p38 MAPK, ERK and JNK, are also activated in DCs when maturation is induced by LPS or tumour necrosis factor-α (Sato *et al.*, 1999; Arrighi *et al.*, 2001). When we analysed MAPK activation in MDDCs stimulated with AM3 or LPS in western blots (Figure 6c), AM3 induced the phosphorylation of p38 MAPK in a time-dependent manner and to a similar extent as seen after LPS. Unlike LPS, which induced strong phosphorylation of ERK, AM3 induced only mild ERK phosphorylation, indicating that AM3 activates similar but not identical pathways to LPS in MDDCs. Together, these observations suggest that AM3-induced maturation could be mediated, at least in part, by p38/NF-κB activation.

AM3 promotes TLR-4-mediated NF-κB activation

To determine whether TLRs play a role in the response of MDDCs to AM3, we examined the effect of AM3 on NF-κB activation in HEK293 cells stably transfected with TLR2 or TLR4. These cells were transfected with the NF-κB-Luc reporter construct, stimulated with different concentrations of AM3 and assayed for luciferase activity. Additionally, cultures were stimulated with either of the purified TLR ligands (Pam3Cys for TLR2 or LPS for TLR4). Cells expressing TLR4 but not TLR2 were capable of activating NF-κB in response to AM3, even though the transfected TLR2 cells were indeed functional and capable of activating NF-κB in response to Pam3Cys (Figure 7a). To confirm whether AM3-mediated MDDC maturation was regulated by TLR4, immature MDDCs were preincubated with anti-TLR-4 or isotype matched antibodies before they were stimulated with AM3 or LPS. TLR-4 antibodies had no effect on the expression of cell surface markers by these cells in the absence of stimuli. As expected, exposure to AM3 and LPS strongly upregulated the surface expression of CD80, CD83 and CD86 in these cells, although preincubating the cells for 30 min with the anti-TLR4 antibody impaired the upregulation of DC maturation-associated markers by both LPS and AM3. As the isotype matched antibody had no effect on these cell surface markers, TLR-4 seemed to be involved in

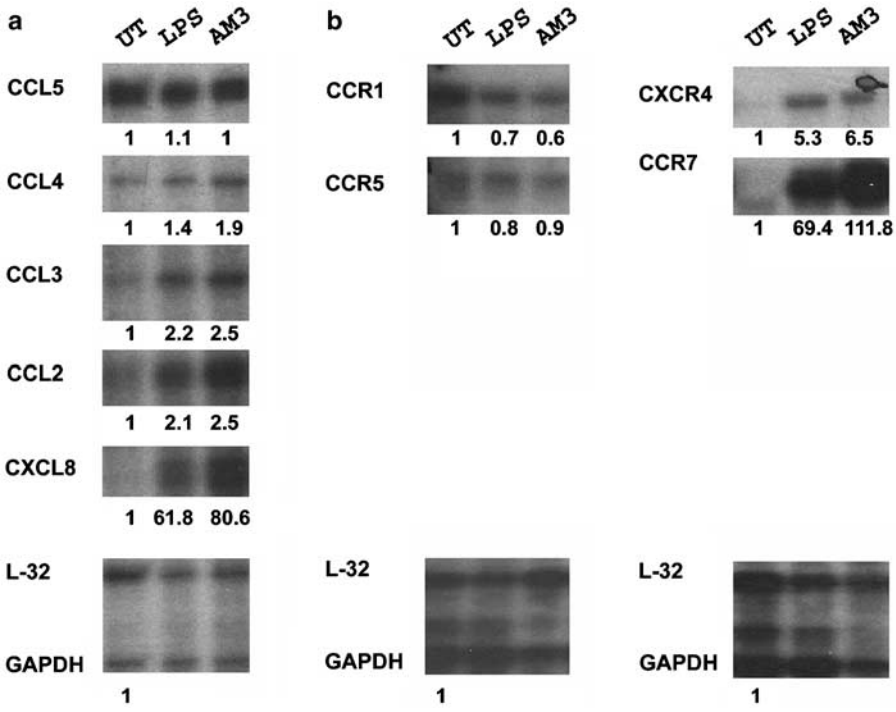

Figure 5 AM3 induces chemokine and chemokine receptor mRNA expression by human immature DCs. Immature MDDCs were either left untreated or they were exposed to 0.1 $\mu g\,mL^{-1}$ LPS or 1 $\mu g\,mL^{-1}$ AM3. After 12 h, expression of mRNA encoding for the indicated chemokines (**a**) or chemokine receptors (**b**) was evaluated in RNase protection assays. The relative levels of chemokine and chemokine receptor mRNA were assessed by scanning densitometry, and the results are expressed in arbitrary units (numbers below the tracks) as the induction over the values obtained in untreated (UT)-MDDCs. Values were normalized to the expression of the ribosomal housekeeping protein L32 in each sample. A representative experiment of three independent donors is shown. DC, dendritic cell; LPS, lipopolysaccharide; MDDC, monocyte-derived DC.

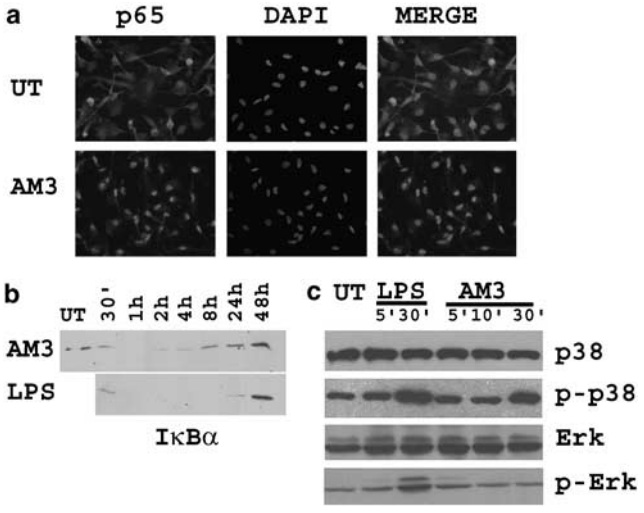

Figure 6 AM3 increases the nuclear localization of p65/reaA, and promotes IkBα degradation and p38 MAPK phosphorylation. (**a**) MDDCs were left untreated (UT) or stimulated with AM3 (1 $\mu g\,mL^{-1}$) for 1 h, and the distribution of p65 was analysed by indirect immunofluorescence (red). The nuclei were stained with DAPI (blue) and the representative fields of one of the two independent experiments are shown. (**b**) MDDCs were either untreated or stimulated with LPS (0.1 $\mu g\,mL^{-1}$) or AM3 (1 $\mu g\,mL^{-1}$) for different times and protein extracts were analysed by western blots probed with anti-IκBα or with (**c**) Anti-phospho-ERK1/2 (p-ERK), anti-ERK1/2 (ERK), anti-phospho-p38 (p-p38), anti-p38 (p38) antibodies. Representative results of three independent experiments performed are shown. LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MDDC, monocyte-derived dendritic cell.

mediating the AM3-induced maturation of MDDCs (Figure 7b).

AM3 promotes MDDC maturation in patients with chronic HCV infection

After 7 days of culture in the presence of granulocyte–macrophage colony-stimulating factor and IL-4, cells from patients were left unstimulated or incubated with AM3 (1 $\mu g\,mL^{-1}$) or LPS (0.1 $\mu g\,mL^{-1}$). The phenotypic markers of MDDCs identified were similar to healthy donors and AM3 induced a marked increase in MHC class II HLA-DR, CD80/B7-1, CD86/B7-2 and CD83 expression (Figure 8a). In mixed lymphocyte reactions (MLRs), we observed that MDDCs exposed to AM3 or LPS promoted the proliferation of allogeneic T cells more strongly than unstimulated cells (Figure 8b). These data demonstrated that AM3 could be useful to promote DC maturation in patients infected with HCV.

Discussion

This study provides further insight into the immunoregulatory activity of AM3 and demonstrates that it induces phenotypic and functional changes in human MDDCs. AM3 upregulated the expression of DC maturation markers in MDDCs including the MHC class II, co-stimulatory and adhesion molecules (HLA-DR, CD86, CD83, CD54). AM3

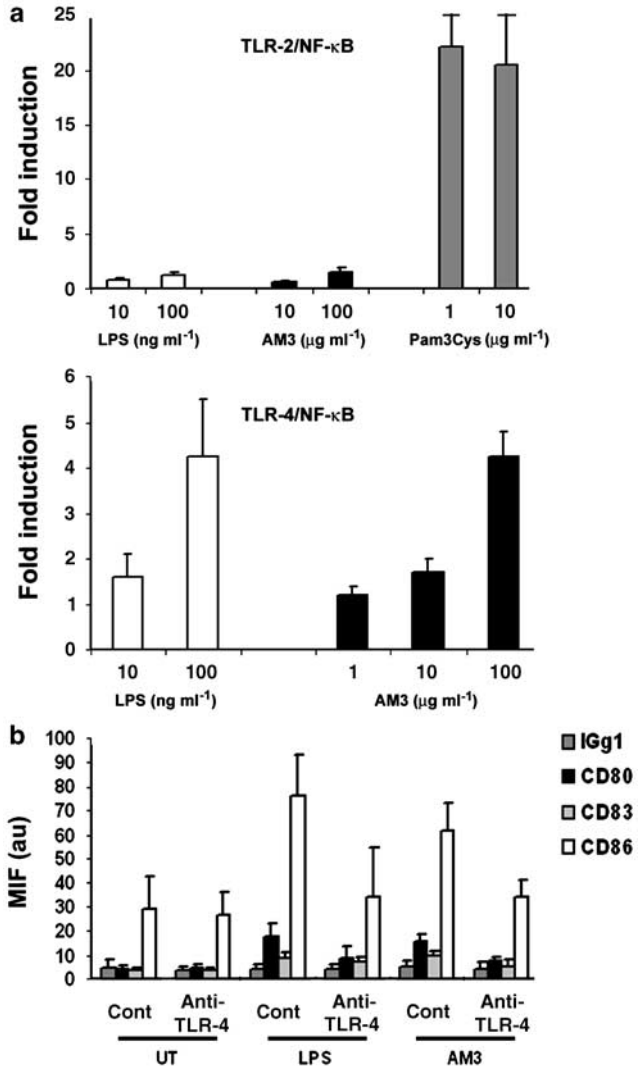

Figure 7 AM3 induces TLR-4-mediated NF-κB activation. (**a**) TLR-4 (upper) and TLR-2 (bottom) stably transfected HEK-293 cells were transfected with κB-Luc plasmid. These cells were exposed to AM3 (1, 10, 100 μg mL^{-1}) and NF-κB activity was evaluated by measuring luciferase levels. The luciferase activity is represented as the induction above the values obtained in the absence of stimulation, and the values are the mean of triplicate experiments (± s.d.). As controls, cells were stimulated with TLR-4 (LPS: 10–100 ng mL^{-1}) and TLR-2 agonists (PamCys3: 1–10 μg mL^{-1}). A representative result from one of the three independent experiments is shown. (**b**) Neutralizing antibodies against TLR4 abolished the upregulation of the surface expression of CD80, CD83 and CD86 induced by AM3 in MDDCs. Cells were left treated with LPS (10 ng mL^{-1}) or AM3 (1 μg mL^{-1}) for 16 h and, additionally, were preincubated with neutralizing anti-TLR4 or isotyped matched antibodies (10 μg mL^{-1}) 1 h before treatment. Cell surface expression was analysed by immunofluorescence. Data are the mean intensity fluorescence (MIF) ± s.d. of four independent experiments. LPS, lipopolysaccharide; MDDC, monocyte-derived dendritic cell; TLR, Toll-like receptor.

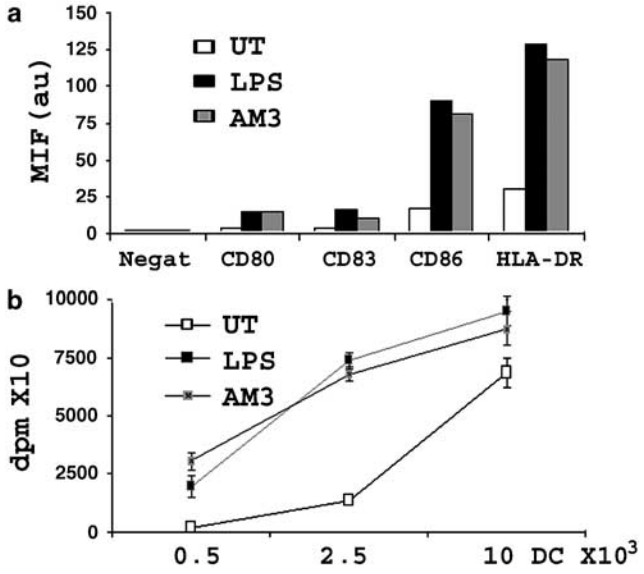

Figure 8 AM3 upregulates cell surface markers and allogeneic T-cell proliferation in MDDCs from chronic HCV-infected patients. (**a**) MDDCs were cultured for 16 h in the presence of AM3 (1 μg mL^{-1}), LPS (0.1 μg mL^{-1}) or medium alone (UT), and the surface markers expressed were analysed by flow cytometry as stated in Materials and methods. The data represent the mean intensity fluorescence (MIF). A representative experiment from one of the eight independent donors is shown. (**b**). MDDCs were cultured in medium alone (UT), with LPS (0.1 μg mL^{-1}) or AM3 (1 μg mL^{-1}) for 24 h. An MLR was conducted for 5 days as described in Materials and methods. The background level of [^{3}H] TdR uptake was determined by measuring the reactions without stimulation. The values are the mean of triplicate experiments (± s.d.). The experiment was carried out on five independent patients and one representative experiment is shown. HCV, hepatitis C virus; LPS, lipopolysaccharide; MLR, mixed lymphocyte reaction; MDDC, monocyte-derived dendritic cell.

also diminished the endocytotic activity of MDDCs while augmenting the capacity of MDDCs to promote the proliferation of allogenic T cells. These data provide further evidence that exposure to AM3 generates functionally active, mature DCs.

Activation of DCs requires priming T cells and DCs are activated by the recognition of a characteristic pattern of pathogens as well as by inflammatory cytokines. Indeed, depending on the stimulus encountered, DCs can induce Th1, Th2 or unpolarized T-cell responses (Kalinski *et al.*, 1999). IL-12 is a fundamental link between the innate and adaptive immune systems, and accordingly IL-12 induces and promotes IFN-γ production in natural killer and T cells and their lytic activity. In addition, IL-12 polarizes the immune system towards a primary Th1 response (Gately *et al.*, 1998). By contrast, IL-10 is a pleiotropic cytokine produced by DCs, T cells and macrophages that has anti-inflammatory and immunosuppressive properties, polarizing the immune system towards a primary Th2 response (Moore *et al.*, 2001). AM3 induces IL-12 production by DCs, and strikingly, the expression of IL-10, possibly due to its ability to bind the DC-specific intercellular adhesion molecule, 3-grabbing non-integrin, at the surface of DCs (Serrano-Gomez *et al.*, 2007). Although they appear conflicting, these results are in accordance with the potentiation of IL-10 and IL-12 expression in DCs induced by LPS (Langenkamp *et al.*, 2000), where upregulating IL-10 limits IL-12 production and controls the inflammatory response (Corinti *et al.*, 2001). Additionally, our data demonstrate that AM3 induces IFN-γ secretion (a pro-Th1 cytokine) without significantly affecting IL-4 production (a pro-Th2 cytokine).

These data suggest that AM3 promotes Th1 polarization of T lymphocytes under the experimental conditions analysed.

Dendritic cell precursors are either constitutively recruited from the bloodstream into tissues or in response to chemotactic signals. Once in the tissues, DCs can be activated by inflammatory cytokines or by bacterial products. These stimuli induce DCs to mature and migrate via the afferent lymph to the T-cell areas of secondary lymphoid organs where they acquire the capacity to stimulate naive T cells (Moser *et al*., 2004). We showed that AM3 upregulated CXCL8, CCL2, CCL3 and CCL4 chemokine expression in MDDCs (Figure 5a), chemokines that are involved in the recruitment of a wide array of cell types including T cells, monocytes, neutrophils and immature DCs (Sallusto *et al*., 1999). Furthermore, DC maturation results in a switch in chemokine receptor expression associated with the downregulation of inflammatory chemokine receptors, including CCR1, and the upregulation of other chemokine receptors, such as CCR7 and CXCR4 (Sallusto *et al*., 1998). Interestingly, AM3 similar to LPS, increases the expression of mRNA for the CCR7 and CXCR4 chemokine receptors. The upregulation of CCR7 is particularly relevant for the homing of mature DCs, as CCR7 ligands are produced in secondary lymphoid organs (Moser *et al*., 2004). These data further support the view that exposure to AM3 promotes the generation of functionally active, mature DCs.

There is growing evidence suggesting that DC maturation is dependent on NF-κB activity. Recent reports have shown that LPS and tumour necrosis factor-α, two potent DC maturation factors, induced NF-κB activation and phosphorylation of p38 in MDDCs (Ardeshna *et al*., 2000; Arrighi *et al*., 2001). Moreover, the p38 MAPK pathway contributes to NF-κB-mediated transactivation. We showed that NF-κB and p38 were activated when immature human DCs were exposed to AM3 and, that these proteins were most likely to be involved in the maturation promoted by AM3.

The mechanism through which AM3 stimulates immature MDDCs was analysed by determining the cell surface receptors induced by AM3. The recognition of pathogens is mediated by a set of germ line-encoded receptors that recognize conserved molecular patterns shared by large groups of microorganisms. TLR play an essential role in the recognition of microbial components and of endogenous ligands induced during innate immune responses (Takeda *et al*., 2003). Two TLRs, TLR2 and TLR4, are implicated in bacterial recognition and they trigger similar cellular transduction pathways, promoting MAPK and NF-κB activation (Re and Strominger, 2001). The interaction of LPS with TLR-4 preferentially activates p38MAPK, whereas TLR-2 ligands, peptidoglycan and bacterial lipoproteins preferentially activate ERK (An *et al*., 2002; Agrawal *et al*., 2003). We show for the first time that AM3, similar to LPS, can interact with TLR4, resulting in the activation of NF-κB. In addition, antibody blockade of TLR-4 impairs both LPS and AM3-induced maturation of DCs. These findings indicate that AM3 might be a TLR4 agonist, which could at least partially account for the effects of AM3 on MDDC maturation. AM3 was recently shown to inhibit the binding of viral, fungal and parasite pathogens to MDDCs (Serrano-Gomez *et al*., 2007). The polysaccharide moiety of AM3 directly influences lectin-dependent pathogen recognition in DCs by interacting with DC-specific intercellular adhesion molecule 3-grabbing nonintegrin. AM3 specifically impairs the pathogen recognition capacity of DC-specific intercellular adhesion molecule 3-grabbing nonintegrin, as it reduces the attachment of *Candida*, *Aspergillus* and *Leishmania* and blocks the DC-specific intercellular adhesion molecule 3-grabbing nonintegrin-dependent capture of HIV virions and the HIV trans-infection capacity (Serrano-Gomez *et al*., 2007).

The effects of chronic hepatitis C on DCs remain somewhat unclear. Initial studies suggested that the activity of DCs was impaired in HCV-infected patients and that these observations could, in part, be a consequence of changes in the surface expression of MHC and co-stimulatory molecules (Kanto *et al*., 1999; Auffermann-Gretzinger *et al*., 2001; Bain *et al*., 2001). However, normal function has also been reported in chronic HCV infection comparable to that in healthy individuals (Longman *et al*., 2004). In our experiments, AM3 enhanced cell surface expression of co-stimulatory and MHC molecules in MDDCs from HCV-infected patients. In addition, the allostimulatory effects of AM3-stimulated MDDCs on T cells were similar to that of LPS-stimulated MDDCs. Our results suggest that AM3 could be useful to promote increased DC activity in patients with chronic HCV infection.

The results presented here may explain some of the *in vivo* effects of AM3. In a classic approach, the plasma levels of a substance are closely related to its pharmacological effects. AM3 is considered as an immunomodulator, one of a heterogeneous group of drugs that are able to act directly on target cells or by modulating the activity of different cell types, thereby exerting their activity through endogenous mediators. In this family of compounds, the relationships between pharmacological effects and plasma levels are very complex. The ubiquitous location of the immune system makes almost all tissues pharmacological targets for drugs that can modulate the immune response. Several studies demonstrated that the components of AM3, both the protein and polysaccharide moiety, are highly resistant to lytic enzymes and denaturing agents (Pantoja-Uceda *et al*., 2002). This could be a crucial factor with regard to their activity after oral administration. The biodistribution of AM3 was analysed after developing a new method for the radioiodination of polysaccharides (Guerrero *et al*., 2000). After oral administration at a dose at which pharmacological effects have been found, no effects on gut motility were found on the basis of the slow transit profile and the fact that both entities show a similar biodistribution (that is, no absorption in the case of the protein and a mild absorption of the polysaccharide, $<5\%$). These data indicate that the pharmacological effects of AM3 can be attributed to its interaction with intestinal tissue, probably through the generation of endogenous mediators by the gut-associated lymphoid tissue (Guerrero *et al*., 2000). Moreover, an increased mitogenic response of Peyer's patches in AM3-treated animals has been observed (unpublished data). It is known that orally administered antigens can be delivered to the DCs located in the mucosa (Rimoldi and Rescigno, 2005). Furthermore, the polysaccharidic component of AM3 can be

detected in the blood (Guerrero *et al*., 2000), indicating that even circulating DCs could interact with this compound. AM3 also acts on peripheral blood mononuclear cells by promoting the release of inflammatory mediators that inhibit Hepatitis B virus replication *in vitro* (Majano *et al*., 2004). Interestingly, using an autonomous and genomic replication system in human hepatoma cells, our previous studies indicate that AM3 stimulates the secretion of molecules with antiviral properties by peripheral blood mononuclear cells, thereby inhibiting HCV replication (our unpublished data). Furthermore, it is suggested that common signalling pathways are activated by AM3 and LPS. In this regard, the adjuvant activity of bacterial products is not only important for antibacterial responses induced by peripheral DCs but also for vaccine development. However, LPS cannot be used owing to its high toxicity, as it is one of the main causative agents of septic shock in humans (Karima *et al*., 1999). In summary, the ability of AM3/Inmunoferon to induce DC maturation in MDDCs derived from healthy and HCV-infected patients, and its lack of systemic toxicity (Brieva *et al*., 2003) suggests that it may be potentially useful as an adjuvant in vaccination protocols in which mature DCs could be used as antigen carriers.

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Conflict of interest

AM3 is an active pharmaceutical ingredient and it is manufactured and commercialized by Dunar, a subsidiary of Industrial Farmaceutica Cantabria. S Gonzalez and JP Pivel are scientific advisers to Industrial Farmaceutica Cantabria. JL Alonso-Lebrero is employed by Industrial Farmaceútica Cantabria SA.

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