

# CHITOSAN DRIVES ANTI-INFLAMMATORY MACROPHAGE POLARISATION AND PRO-INFLAMMATORY DENDRITIC CELL STIMULATION

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## Abstract

Macrophages and dendritic cells (DC) share the same precursor and play key roles in immunity. Modulation of their behaviour to achieve an optimal host response towards an implanted device is still a challenge. Here we compare the differentiation process and polarisation of these related cell populations and show that they exhibit different responses to chitosan (Ch), with human monocyte-derived macrophages polarising towards an anti-inflammatory phenotype while their DC counterparts display pro-inflammatory features. Macrophages and DC, whose interactions with biomaterials are frequently analysed using fully differentiated cells, were cultured directly on Ch films, rather than exposed to the polymer after complete differentiation. Ch was the sole stimulating factor and activated both macrophages and DC, without leading to significant T cell proliferation. After 10 d on Ch, macrophages significantly down-regulated expression of pro-inflammatory markers, CD86 and MHCII. Production of pro-inflammatory cytokines, particularly TNF- $\alpha$ , decreased with time for cells cultured on Ch, while anti-inflammatory IL-10 and TGF- $\beta$ 1, significantly increased. Altogether, these results suggest an M2c polarisation. Also, macrophage matrix metalloproteinase activity was augmented and cell motility was stimulated by Ch. Conversely, DC significantly enhanced CD86 expression, reduced IL-10 secretion and increased TNF- $\alpha$  and IL-1 $\beta$  levels. Our findings indicate that cells with a common precursor may display different responses, when challenged by the same biomaterial. Moreover, they help to further comprehend macrophage/DC interactions with Ch and the balance between pro- and anti-inflammatory signals associated with implant biomaterials. We propose that an overall pro-inflammatory reaction may hide the expression of anti-inflammatory cytokines, likely relevant for tissue repair/regeneration.

**Keywords:** Macrophage; dendritic cell; chitosan; polarisation; cell activation; immunomodulation; inflammation; immune response.

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## Introduction

Understanding the biological events taking place at implant surfaces is of key relevance for the development of successful and functional biomaterials. A multitude of host cell populations respond to implantation, both at short and long-term, and the inter-communication established will depend on the biomaterial characteristics. Immune cells, in particular macrophages, mediate a great number of responses, ranging from initial inflammation to tissue remodelling and regeneration, through clearance of exogenous agents and production of cytokines and chemokines that activate/deactivate or recruit other surrounding inflammatory or wound healing cells to the injury site (Xia and Triffitt, 2006). Besides their role as inflammatory mediators, these phagocytic cells have also the remarkable characteristic of presenting antigens to T cells, thereby initiating the mechanisms of adaptive immunity. Depending on the micro-environmental stimuli received, macrophages can display either pro- or anti-inflammatory features, performing distinctive functions. Classically or pro-inflammatory-activated macrophages, also designated as M1, can be primed by interferon  $\gamma$  (INF $\gamma$ ) and/or lipopolysaccharides (LPS) and have high microbicidal activity, with increased production of pro-inflammatory cytokines. On the other hand, alternatively-activated M2 macrophages, primed by interleukin (IL)-4 (IL-4) and IL-13 (M2a subset), immune complexes and LPS (M2b subset), or IL-10 (M2c subset), are associated with an anti-inflammatory profile and are crucial in tissue remodelling after inflammation (Mantovani *et al.*, 2005; Mantovani *et al.*, 2004).

In addition to macrophages, another immune cell type with key functions in immunity is the dendritic cell population. They are specialised in the up-take, processing and presentation of antigens, including implant-associated antigens (Babensee *et al.*, 1998) to T lymphocytes and are considered professional antigen presenting cells (APC) (Banchereau *et al.*, 2000). Similarly to macrophages, dendritic cells (DC) also show some degree of plasticity, depending on the external stimuli. Inflammatory or classical mature DC can be activated by foreign antigens, produce pro-inflammatory cytokines and stimulate T cells, whereas tolerogenic and regulatory DC can be activated by self-antigens or anti-inflammatory molecules, release anti-inflammatory cytokines and induce the expansion of regulatory T cells, in a striking resemblance with M1 and M2 macrophages (Kou and Babensee, 2011). Despite sharing the same precursor, the myeloid-monocyte haematopoietic cell, as well as significant overlapping

functions, macrophages and DC display different cell surface expression molecules, particularly upon activation. Furthermore, the innate cytotoxicity of macrophages and their ability to fuse forming foreign body giant cells renders them unique (Kou and Babensee, 2011). As these two cell types bridge innate and adaptive immunity, guiding the host response, understanding their cellular behaviour to a specific material is of foremost importance for effective applicability of devices in a tissue engineering perspective. Moreover, this knowledge is crucial for the development of new biomaterial-based strategies that, through modulation of the macrophage/DC phenotype towards reparative/tolerogenic functions, could enhance tissue regeneration. In this context, a comparative investigation of both human monocyte-derived macrophages and DC differentiation and maturation directly on the same biomaterial was carried out and is presented herein. The natural and biocompatible polymer chitosan (Ch), intensively investigated for a wide range of biomedical applications, such as drug delivery, wound dressings, and tissue engineering strategies (Baldrick, 2010; Dash *et al.*, 2011; Kim *et al.*, 2008; Muzzarelli, 2009; Ueno *et al.*, 2001a), was selected and presented to cells as an ultrathin matrix on which macrophage and DC differentiation took place during 10 or 7 d, respectively. This approach was selected since biomaterial surfaces have been shown to affect macrophage phenotype (Ariganello *et al.*, 2011; Dinnes *et al.*, 2008; Jones *et al.*, 2007), and because Ch, reported as immuno-stimulatory (Mori *et al.*, 1997; Peluso *et al.*, 1994; Porporatto *et al.*, 2003; Ueno *et al.*, 2001b), is frequently presented to already differentiated cells (Mori *et al.*, 2005; Peluso *et al.*, 1994; Porporatto *et al.*, 2003), possibly underestimating its potential to modulate immune cell behaviour during the inflammatory and later repair processes. The host-tissue response to Ch-based implants has been investigated with contradictory results. Some studies have documented Ch as a material that evokes a minimal foreign body reaction (Azab *et al.*, 2007; Yussuf *et al.*, 2011), which would render it suitable for tissue engineering. No evidence of exacerbated inflammatory reaction to the material itself was found for different species and implantation sites, including Ch (degree of acetylation (DA) = 15 %) implants in the spinal cord of rats, in comparison with Vicryl and Gore-Tex (Kim *et al.*, 2011), intraperitoneal and subcutaneous implantation of a Ch (DA = 8 %) scaffold in mice (VandeVord *et al.*, 2002) and subcutaneous implantation in rats of a Ch (DA = 11 %) bilayer relative to two commercially available skin substitutes (Yussuf *et al.*, 2011). Conversely, others found a pronounced inflammatory response induced by Ch, as in the case of Ch (DA = 15 %) injection to the brain in rats (Crompton *et al.*, 2006) and subcutaneous implantation of Ch (DA = 15 %) samples in rats (Peluso *et al.*, 1994). These contradictory data are quite often related to purity, contaminants and differences in the DA of the polymer. In fact, it has been shown that the inflammatory reaction to Ch can be reduced by decreasing its DA (Barbosa *et al.*, 2010; Hidaka *et al.*, 1999; Kim *et al.*, 2011; Tomihata and Ikada, 1997).

In this investigation, macrophages and DC were cultured directly on Ch films, rather than exposed to the

polymer after complete differentiation. Ch was the sole stimulating factor and its role on macrophage polarisation and DC stimulation was studied.

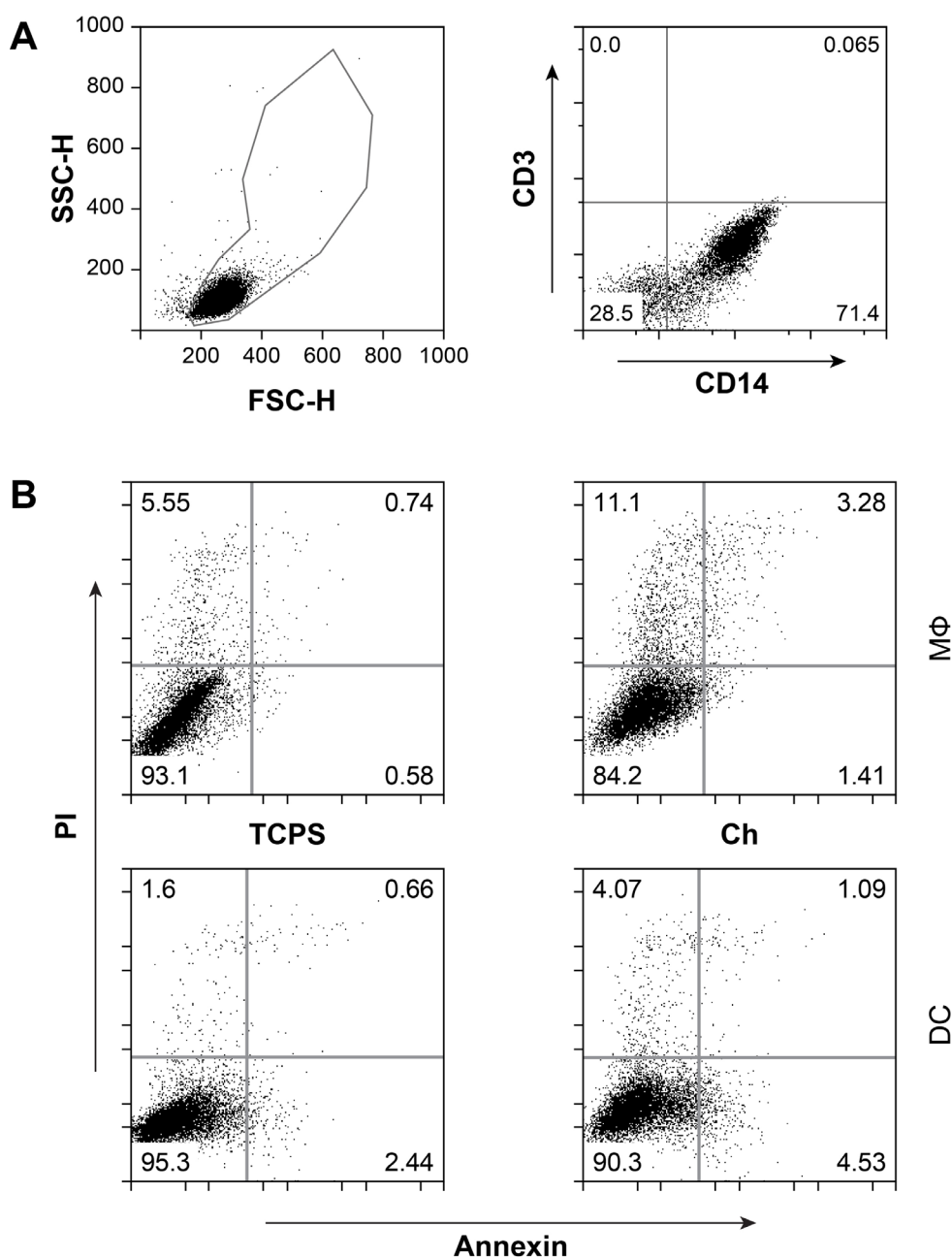
## Materials and Methods

### Chitosan films

High molecular weight (Mw) chitosan (France Chitine, Orange, France, degree of N-acetylation of 11–12 %, Mw  $324 \pm 27 \times 10^3$ , endotoxin-free) was hydrated using Milli-Q water, and then dissolved in 0.1 M HCl under stirring and filtered. Ch was next precipitated with 0.1 M KOH, washed with Milli-Q water, freeze-dried (48 h) and finally milled (IKA, Königswinter, Germany) until a fine powder was obtained. After purification Ch was dissolved in 0.2 M acetic acid and the solution (0.5 %) degassed, filtered through 0.2  $\mu$ m pore filters (Millipore, Billerica, MA, USA) and stored at 4 °C protected from light. As substrate, glass coverslips, previously washed overnight with 65 % nitric acid, washed several times with distilled water and calcinated overnight at 220 °C, were used. Ch films of  $24 \pm 4$  nm thickness (measured by ellipsometry; data not shown) were prepared by spincoating (2 min at 9000 rpm) on these glass coverslips. After spinning, films were neutralised with 0.1 M NaOH for 5 min, washed twice with MilliQ water and dried for 24 h at 37 °C. Prior to cell culture, films were sterilised with filtered 70 % ethanol, washed twice with phosphate-buffered saline (PBS, Sigma-Aldrich Inc., St. Louis, MO, USA) and incubated with complete medium for 1 h at 37 °C.

### Cell culture

Human primary monocytes were obtained from buffy coats (BC) from healthy blood donors, kindly donated by Instituto Português do Sangue, using the *RosetteSep* human monocyte enrichment isolation kit (StemCell Technologies SARL, Grenoble, France). Briefly, BC were centrifuged at room temperature for 20 min at 1200 g without active brake. The peripheral blood mononuclear cell (PBMC) layer was collected and incubated with *RosetteSep* human monocyte enrichment isolation kit according to the manufacturer's instructions. The mixture was then diluted at a 1:1 ratio with PBS, 2 % foetal bovine serum (FBS, Lonza, Basel, Switzerland) before being layered over Histopaque®-1077 (Sigma-Aldrich) and centrifuged as previously. The enriched monocyte layer was collected carefully and washed with PBS. The washes were performed at 700 rpm for 17 min to ensure platelet removal. Finally, the pellet was resuspended in complete culture medium (CM-RPMI 1640 + Glutamax (Invitrogen, Paisley, UK)) supplemented with 10 % FBS and 1 % penicillin G-streptomycin (Invitrogen). The recovered population routinely contained > 70 % of CD14 positive cells and no other detectable cell population (Fig. 1A). Cells were then counted in a Neubauer chamber using the trypan blue dye (Sigma-Aldrich)-exclusion assay, plated on Ch films or tissue culture polystyrene (TCPS) plates (BD Biosciences, Franklin Lakes, NJ, USA) and cultured in a humidified 37 °C/5 % CO<sub>2</sub> incubator for different periods of time. Monocytes were allowed to differentiate



**Fig. 1.** Cell population purity and viability. Cells were obtained from buffy coats and monocyte isolation performed by negative selection using *RosetteSep* monocyte enrichment kit. **(A)** Aspect ratio (left panel) and CD3 (T cell marker) and CD14 (monocyte lineage marker) double staining (right panel) of cells immediately after purification. **(B)** Viability of macrophages (top panels) and DC (bottom panels) was analysed by Annexin/PI staining after 10 or 7 d of culture, respectively, on TCPS (left panels) and Ch (right panels). MΦ, macrophages; DC, dendritic cells; TCPS, tissue culture polystyrene; Ch, chitosan; PI, propidium iodide; FSC, forward scatter; SSC, side scatter.

into macrophages, without addition of cytokines, for 3, 7 or 10 d as commonly performed in macrophage-biomaterial studies (Anderson and Jones, 2007; Jones *et al.*, 2007; Jones *et al.*, 2008). Monocyte-derived DC were differentiated as described previously (Chapuis *et al.*, 1997; Sallusto and Lanzavecchia, 1994). Briefly, monocytes were cultured for 7 d with IL-4 (50 ng/mL) and GM-CSF (50 ng/mL) (both from Immunotools, Friesoythe, Germany) to obtain fully differentiated immature DC. Cell differentiation was assessed by cell surface labelling for lineage markers, CD14 for macrophages or CD1a for DC

(Chapuis *et al.*, 1997; Kou and Babensee, 2011), and flow cytometry. To induce macrophage polarisation into pro-inflammatory M1 or anti-inflammatory M2c phenotypes, cells cultured on TCPS were treated at day 7 with 10 ng/mL lipopolysaccharide (LPS) or 10 ng/mL IL-10, respectively, and left in culture for an additional 3 d. This protocol was adapted from a previously described method (Porcheray *et al.*, 2005). To promote DC maturation, cells differentiated on TCPS were treated with 50 ng/mL LPS for 24 h (Santos *et al.*, 2008; Verhasselt *et al.*, 1997). Purified T cells were isolated from BC by centrifugation (800 g for 30 min at



room temperature, without active brake) over Lymphoprep (Axis-Shield PoC, Oslo, Norway) followed by negative isolation using magnetic beads (*Easysep* T-cell negative isolation kit, StemCell Technologies), according to the manufacturer's instructions. CD3 positive T lymphocytes were consistently over 90 % of the cells purified using this negative selection method. PBMC were obtained from BC following Lymphoprep density gradient centrifugation as above, and plated on TCPS petri dishes at  $5 \times 10^6$  cells/mL and lymphocytes separated from the monocytic population by a 2 h adherence step. All cells were cultured in complete culture medium. At the appropriated time points, macrophages and DC cultured on the different substrates were harvested after 20–30 min incubation at 37 or 4 °C with PBS-5 mM EDTA (Sigma-Aldrich) prior to experiments.

### Flow cytometry

For cell surface staining analysis, freshly isolated monocytes were plated at  $0.1 \times 10^6$  cells/cm<sup>2</sup> or  $0.2 \times 10^6$  cells/cm<sup>2</sup> and allowed to differentiate into macrophages or DC, respectively. Fully differentiated cells, macrophages at day 10 and DC at day 7, were gently harvested, washed and resuspended in staining buffer (PBS, 2 % FBS, 0.01 % azide). Labelling was performed in a final volume of 50 µL with the indicated fluorescently conjugated antibodies for 45 min at 4 °C in the dark. Cells were then washed three times with staining buffer and transferred to FACS tubes for analysis by flow cytometry. The following antibodies were used: anti-CD1a (PE and APC), anti-CD3 (FITC and PE), anti-CD4-APC, anti-CD8-PE, anti-CD14 (FITC, PE and APC), anti-CD86-FITC, HLA-DR-PE (all from Immunotools, Friesoythe, Germany), anti-CD69-FITC (Dako, Glostrup, Denmark), anti-CD83-FITC (AbD Serotec, Kidlington, UK), anti-CD163-PE and anti-CD206-Cy5 (both from R&D Systems, Minneapolis, MN, USA). Isotype matched antibodies were used as negative controls, to define background staining. To assess cell viability, macrophages and DC were first stained, as described above, for the lineage markers CD14 or CD1a, respectively, then incubated with Annexin V-FITC and propidium iodide for 15 min (BD Biosciences) and finally analysed by flow cytometry. Fluorescence was measured using a FACS Calibur flow cytometer (BD Biosciences) with Cell Quest software, and 10,000 events were collected per sample. Analysis was performed using FlowJo software. Mean fluorescence intensity (MFI) values were calculated by subtracting the respective isotype control.

### Cytokine production

For cytokine evaluation, freshly isolated monocytes were seeded at  $0.125 \times 10^6$  cells/cm<sup>2</sup> on TCPS or Ch films and cell culture supernatants from 3-, 7- and 10-day differentiated macrophages and 7-day differentiated DC, on both substrates, were collected, centrifuged to remove cell debris, and assayed for IL-1 $\beta$ , IL-6, IL-10, IL-23, TNF- $\alpha$  and TGF- $\beta$ 1 production by ELISA (Legend Max Human ELISA kits, all from BioLegend, San Diego, CA, USA), according to the manufacturer's protocol. IL-12 production was determined using the Quantikine anti-human IL-12-immunoassay (R&D Systems). Sample concentrations (pg/

mL) were determined from the mean absorbance values for each set of samples, compared to a standard calibration curve. The examination of DC cell culture supernatants at shorter time points was not performed, as these cells require 5–7 d to fully differentiate (Babensee and Paranjpe, 2005; Chapuis *et al.*, 1997).

### Gelatin zymography

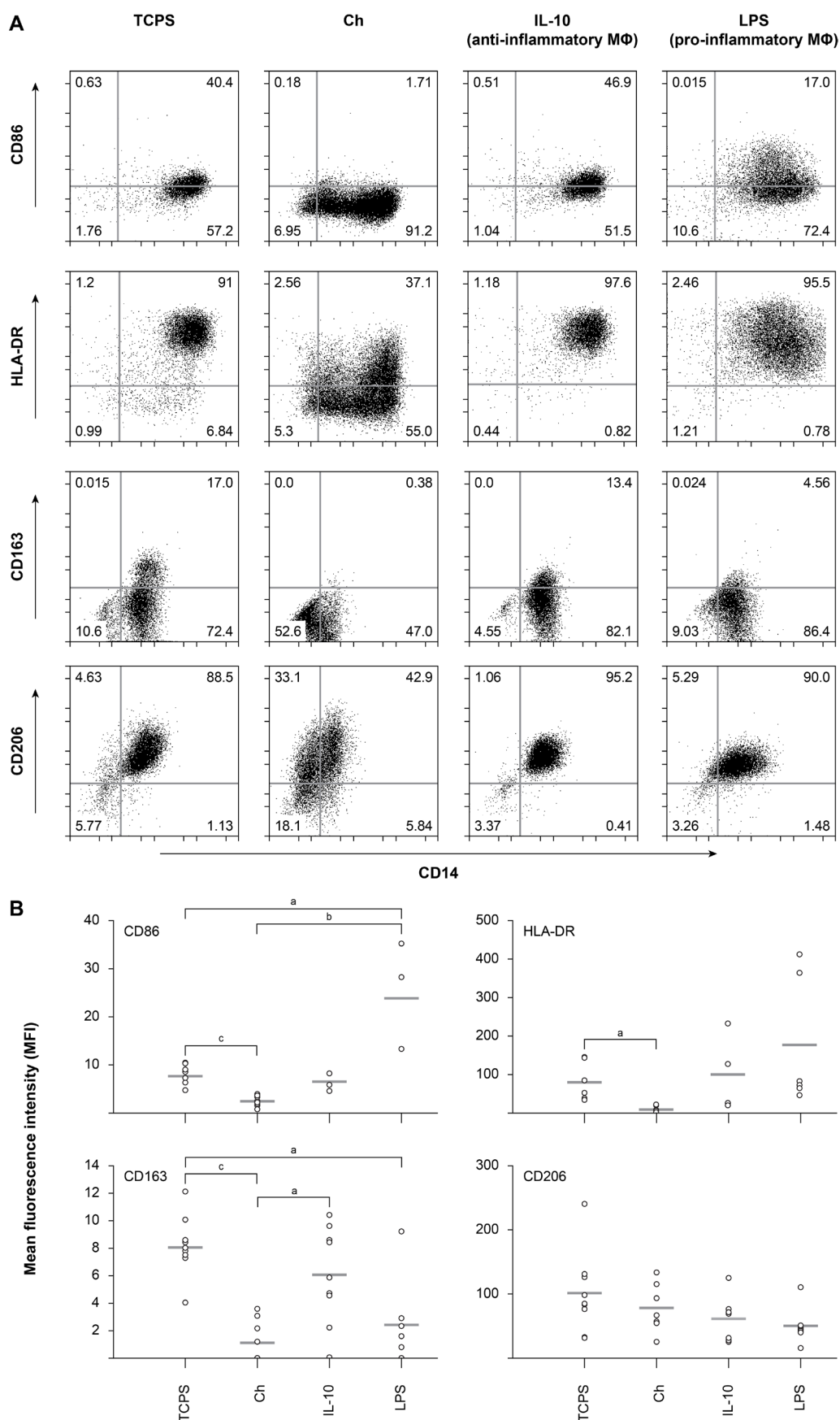
The same cell culture supernatants used for ELISA were analysed by gelatin zymography in 0.1 % gelatin type B (Sigma-Aldrich) -10 % polyacrylamide- (Bio-Rad, Hercules, CA, USA) gels. 10 or 20 µg protein (for macrophages or DC, respectively) were prepared in SDS sample buffer and run, without denaturation, at 60 V, 100 mA. After electrophoresis, gels were washed twice with 2 % Triton X-100 (Sigma-Aldrich) for 15 min on an orbital shaker to remove SDS remnants. Gels were then incubated for 16 h in appropriate matrix metalloproteinase (MMP) substrate buffer, washed in distilled water, stained with Coomassie Brilliant Blue R-250 solution (Sigma-Aldrich) for approximately 30 min and finally transferred to distilled water. MMP activity was quantified by calculating for each band the pixel intensity per mm<sup>2</sup>, using the QuantityOne software (Bio-Rad).

### Cell motility

Cell movement was evaluated on both Ch films and TCPS and monitored for 12 h using time-lapse video microscopy. Macrophages differentiated for 3, 7 or 10 d and DC differentiated for 7 d in TCPS were gently detached with PBS-EDTA, and allowed to adhere to Ch films or TCPS for 2 h at 37 °C and 5 % CO<sub>2</sub> atmosphere. Bright field images were then captured on 5 distinct microscopic areas every 5 min using a 20 x objective of an automated inverted Zeiss Zx2 microscope (Carl Zeiss, Oberkochen, Germany) and a digital charge-coupled camera. A time-lapse sequence was formed by merging individual images and 12 cells, at least, were randomly chosen for quantification of distance travelled. Cell tracking was performed using FiJi software.

### Analysis of T cell proliferation

Monocytes isolated as described above were plated on Ch films or TCPS at  $0.03 \times 10^6$  cells/cm<sup>2</sup>, with or without differentiating cytokines, for DC or macrophages, respectively. T lymphocytes were counted with a Neubauer chamber and the required number of cells was diluted for  $1 \times 10^7$  cells/mL and labelled with 1 µM carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen) at 37 °C for 15 min in PBS, followed by two washes (5 min at 2500 rpm) with PBS supplemented with 20 % FBS for fluorescence quenching. T cells were then resuspended in complete medium and  $0.9 \times 10^6$  cells were added to each well containing  $0.3 \times 10^6$  monocytes (3:1 ratio) (Huber *et al.*, 2010; Larsen *et al.*, 1994; Mangi and Kantor, 1975; Scheinecker *et al.*, 1998), or left alone on the substrates in presence or absence of the same cytokines used to differentiate DC. Also, peripheral blood lymphocyte samples from the same donor as purified T cells were stimulated with phytohaemagglutinin (PHA) (Sigma-Aldrich), a mitogenic agent, as a positive proliferation control. After 7 d of co-culture, cells were harvested



**Fig. 2.** Expression of cell surface markers of macrophages differentiated on Ch films. **(A)** Human primary monocyte-derived macrophages, differentiated for 10 d, were harvested and cell surface stained with the indicated antibodies before FACS analysis. LPS or IL-10 stimulation was also performed on cells cultured on TCPS for control purposes. **(B)** Scatter charts represent the mean fluorescence intensity (MFI) for each cell surface marker from, at least, 3 different donors. Superscripts indicate statistically significant difference ( $^a p < 0.05$ ;  $^b p < 0.01$ ;  $^c p < 0.001$ ). MΦ, macrophages; TCPS, tissue culture polystyrene; Ch, chitosan; IL-10, interleukin-10; LPS, lipopolysaccharide.

and cell surface-labelled, as described above, with anti-CD3-PE conjugated antibodies to evaluate specific T cell proliferation. Unlabelled T lymphocytes were used to perform isotype control stainings. A single CD3-PE staining, and cells labelled with CFSE alone were used as single stainings, to define sample compensation. Samples were acquired in a FACS Calibur (BD Biosciences) using Cell Quest software, and further analysed with FlowJo software. T cell division was determined by the extent of CFSE halving on CD3 positive cells.

### Statistical analysis

Statistical analysis was performed using Prism5 software, v5.0a. Results were analysed for Gaussian distribution using the D'Agostino and Pearson normality test and samples were non-parametric. The Mann-Whitney test was used to compare two samples, whereas comparison between more than two samples was performed using the Kruskal-Wallis test followed by Dunns comparison test. A value of  $p < 0.05$  was considered statistically significant: <sup>a</sup> $p < 0.05$ ; <sup>b</sup> $p < 0.01$ ; <sup>c</sup> $p < 0.001$ . Data are presented showing all individual donors and the respective mean, or as mean  $\pm$  SD.

## Results

### Chitosan stimulates both macrophages and DC

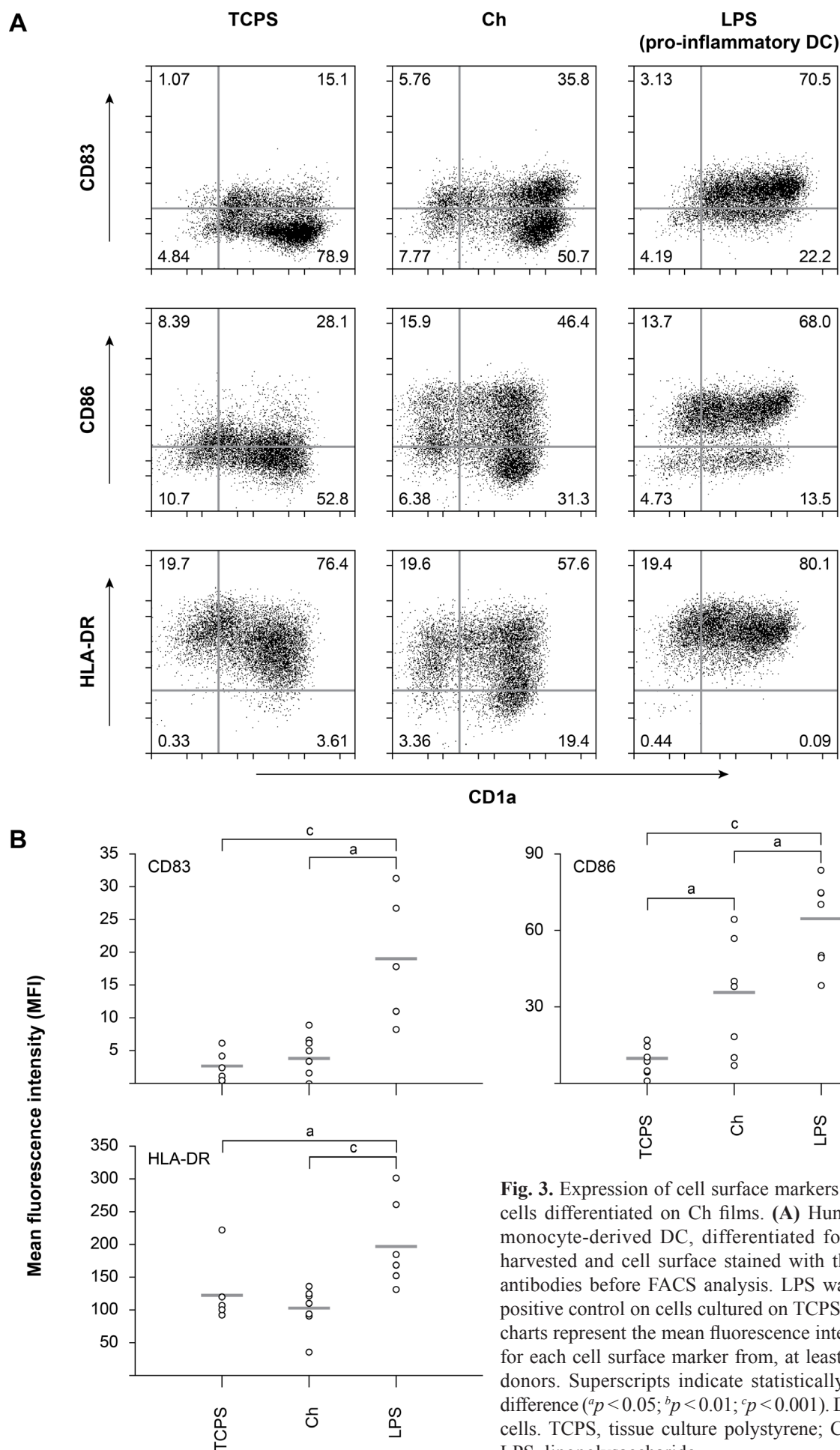
The potential of Ch to act as an immunostimulatory substrate, via its influence on macrophages and DC, was investigated by allowing the differentiation of their precursors, monocytes, directly on Ch films. Isolation of monocytes by negative selection resulted on a highly enriched CD14<sup>+</sup> monocytic population, with no detectable contamination by CD3<sup>+</sup> T lymphocytes (Fig. 1A). This ensured that the cellular responses analysed are cell type specific, and not the result of intercellular crosstalk. DC and macrophage differentiation were allowed to take place for 7 or 10 d, respectively, on Ch films or control TCPS. As shown in Fig. 1B, both cell types displayed high cell viability on Ch substrates. In addition, positive controls of LPS stimulation to induce DC maturation and IL-10 or LPS treatment to commit macrophages into anti-inflammatory (M2c) or pro-inflammatory (M1) phenotypes, respectively, were also performed with cells cultured on TCPS. Importantly, no exogenous cytokines were added to promote macrophage differentiation. The ability of Ch to modulate macrophage polarisation was evaluated by cell surface expression of specific phenotypic markers for human polarised macrophages, together with the lineage marker CD14. Dot plots from a representative experiment are depicted in Fig. 2A, and the MFI, calculated over at least 3 different biological replicates is presented in Fig. 2B. After 10 d of differentiation on Ch films, macrophages significantly down-regulated ( $p < 0.05$ ) the expression of the M1 phenotypic markers CD86 and MHCII (HLA-DR), in contrast to that observed following LPS stimulation (Fig. 2A and B). Intriguingly, when the M2 phenotypic markers CD163 (scavenger receptor) and CD206 (mannose receptor) were evaluated, a decrease in the expression levels of CD163 was also observed on

Ch in comparison with cells cultured on TCPS. In fact, very few macrophages differentiated on Ch expressed CD163, whereas a significant percentage still retained CD206 expression at the cell surface (Fig. 2A and B). This was also accompanied by a decrease in the levels of the monocytic lineage marker CD14, for cells cultured on Ch, when compared with TCPS (Fig. 2A), which has been associated with the progression of macrophage differentiation (Steinbach and Thiele, 1994), indicating that this polymer may accelerate macrophage maturation. In agreement with previous reports, both CD163 and CD206 displayed high basal levels of expression, which were differentially affected by IL-10 or LPS stimuli (Fig. 2B); while LPS induced a significant decrease in cell surface expression of CD163 ( $p < 0.05$ ), CD206 was not significantly altered by LPS or IL-10 (Porcheray *et al.*, 2005). Statistically significant differences between cells cultured on Ch relative to culture on TCPS were found for the expression of CD86 ( $p < 0.001$ ), HLA-DR ( $p < 0.05$ ), and CD163 ( $p < 0.001$ ).

Besides macrophages, monocytes can also differentiate into DC, another cell population with a key role in innate and adaptive immunity. Thus, the activation status of Ch-differentiated DC was next evaluated by measuring the up-regulation of a classical DC activation marker, CD83, as well as of the co-stimulatory molecule CD86 and the MHCII molecule HLA-DR. In addition, CD1a was used as a DC lineage marker. As shown in Fig. 3, Ch induced a statistically significant ( $p < 0.05$ ) up-regulation of CD86 expression on cells from six different donors. As expected, LPS induced a greater up-regulation of this cell surface molecule ( $p < 0.001$ ) (Fig. 3A and B). Although Ch induced a partial up-regulation of CD83 and a decrease of HLA-DR expression, as illustrated in Fig. 3A, no statistically significant differences were found for the levels of expression of both markers on Ch, when compared with TCPS control, as opposed to LPS stimulation, which induced significant increases of CD83 and MHCII expression on DC cultured on TCPS ( $p < 0.001$  for CD83 and  $p < 0.05$  for MHCII) (Fig. 3B).

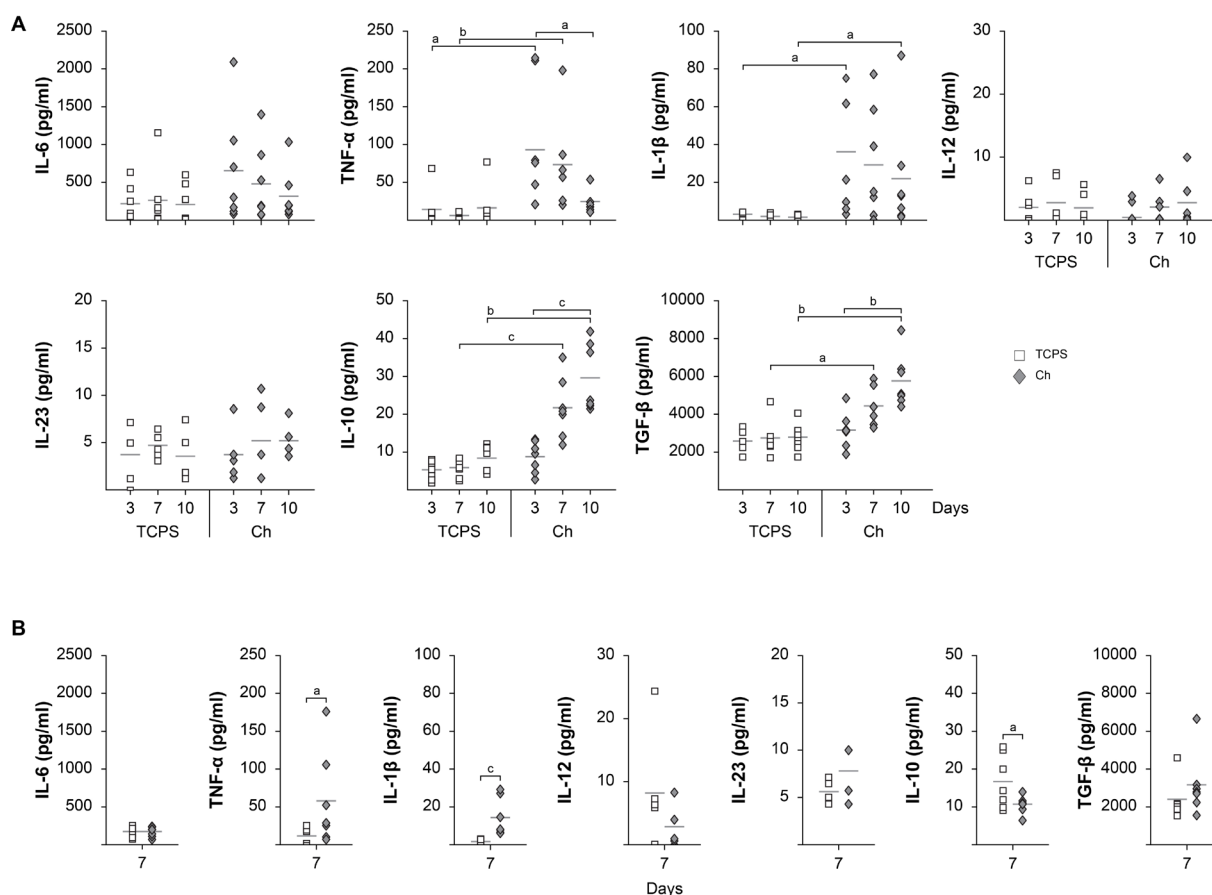
### Chitosan modulates macrophage and DC cytokine production pattern

To better understand how Ch affects the differentiation and activation of monocyte-derived cells, the cytokines produced by both macrophages and DC differentiated on this substrate were evaluated across at least 6 different individuals. The cytokine profile for M1 and M2 macrophage phenotypes has been established, and together with the expression pattern of cell surface molecules, can be used to characterise different population subsets. When macrophages were cultured on Ch, they increased the production of pro-inflammatory cytokines, at early differentiation stages, in comparison with control TCPS conditions (Fig. 4A). Levels of both TNF- $\alpha$  and IL-1 $\beta$  were significantly higher at day 3, for cells cultured on Ch than on TCPS. However, if we compare only cells cultured on Ch with time, there is a reduction of the levels of all pro-inflammatory cytokines tested, with TNF- $\alpha$  being statistically lower between days 3 and 10 of culture (Fig. 4A). Secretion of pro-inflammatory IL-12 and IL-



**Fig. 3.** Expression of cell surface markers of dendritic cells differentiated on Ch films. **(A)** Human primary monocyte-derived DC, differentiated for 7 d, were harvested and cell surface stained with the indicated antibodies before FACS analysis. LPS was used as a positive control on cells cultured on TCPS. **(B)** Scatter charts represent the mean fluorescence intensity (MFI) for each cell surface marker from, at least, 6 different donors. Superscripts indicate statistically significant difference (<sup>a</sup> $p < 0.05$ ; <sup>b</sup> $p < 0.01$ ; <sup>c</sup> $p < 0.001$ ). DC, dendritic cells. TCPS, tissue culture polystyrene; Ch, chitosan; LPS, lipopolysaccharide.





**Fig. 4.** Cytokine production by Ch-differentiated macrophages and DC. Cytokine release was determined by ELISA, using cell culture supernatants from 3, 7 or 10 d differentiated human primary monocyte-derived macrophages (A) or 7 d differentiated human primary monocyte-derived DC (B). Superscripts indicate statistically significant difference (<sup>a</sup> $p < 0.05$ ; <sup>b</sup> $p < 0.01$ ; <sup>c</sup> $p < 0.001$ ). Open squares represent TCPS samples while grey diamonds correspond to chitosan samples. Responses were determined from cells, of at least, 6 different donors. TCPS, tissue culture polystyrene; Ch, chitosan.

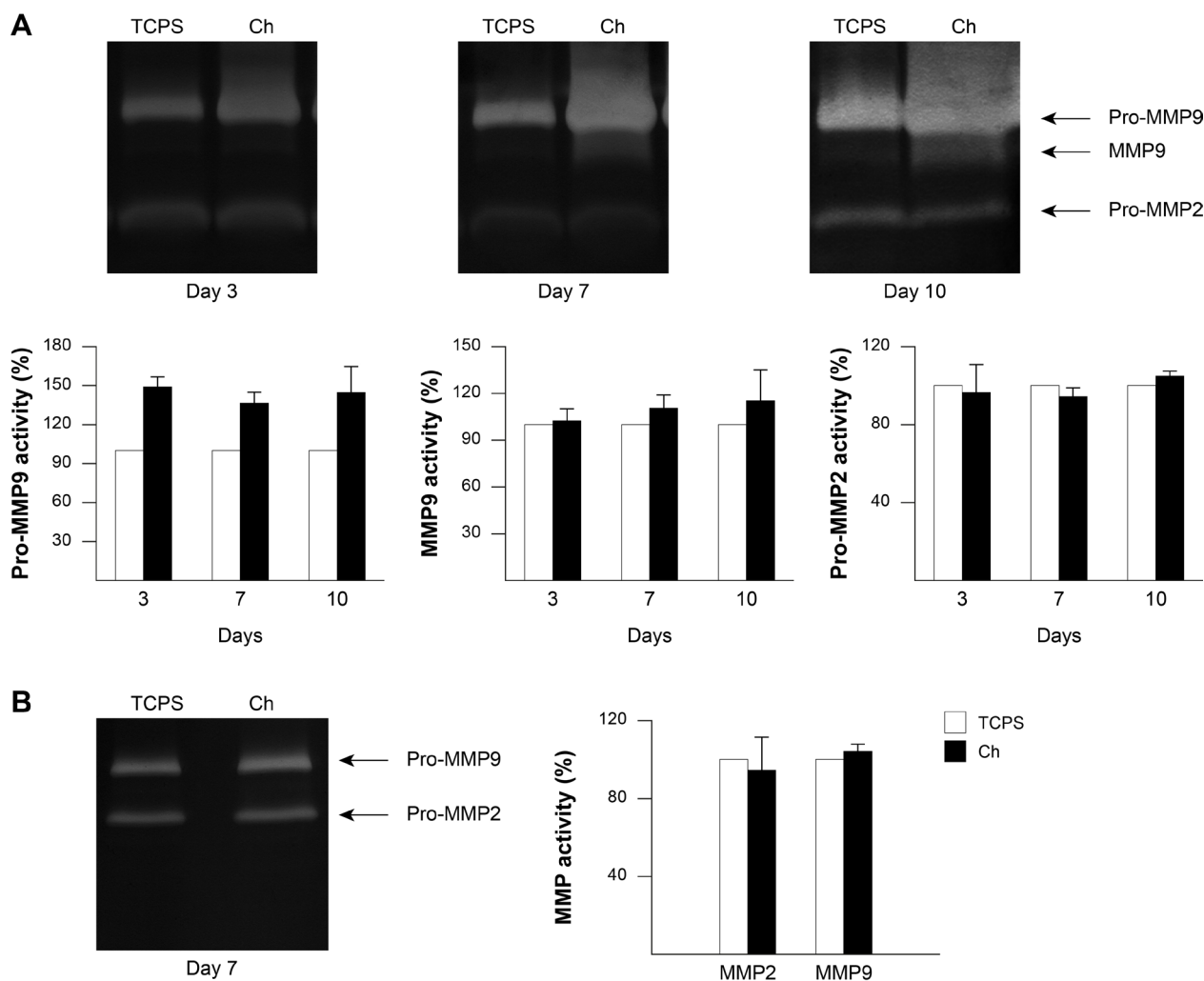
23 on Ch was low during the entire culture period (Fig. 4A). In contrast, Ch induced a significant up-regulation of anti-inflammatory cytokines IL-10 and TGF- $\beta$ 1 at later differentiation stages, and when compared to TCPS. Importantly, in contrast to the gradual reduction observed for anti-inflammatory cytokine release during macrophage differentiation on Ch films, the levels of both IL-10 and TGF- $\beta$ 1 production augmented with increasing times on Ch (3.5 fold increase for IL-10 and 1.8 fold increase for TGF- $\beta$ 1 from day 3 to day 10) (Fig. 4A).

Although IL-6 release by DC was not affected by the substrate (Fig. 4B), Ch significantly stimulated the production of pro-inflammatory cytokines TNF- $\alpha$  ( $p < 0.05$ ) and IL-1 $\beta$  ( $p < 0.001$ ), with levels 4.6 and 11.4 fold higher than on TCPS, respectively (Fig. 4B). This was in clear contrast to the pattern observed for the same cytokines on fully differentiated macrophages, i.e. at day 10 (Fig. 4A). Moreover, unlike macrophages, Ch-differentiated DC significantly down-regulated the secretion of IL-10 in comparison with TCPS. Nonetheless, IL-12 and IL-23 were found to be residual for both cell types independently of the substrate used. Finally, like macrophages, DC increased the secretion of TGF- $\beta$ 1 when cultured on Ch, albeit to a lesser extent.

#### Chitosan enhances the activity of MMP9 released by macrophages, but not by DC

Cells of the monocyte/macrophage lineage, including DC, are active producers of MMPs (Goetzl *et al.*, 1996; Webster and Crowe, 2006). Thus, in order to further characterise the populations of macrophages and DC present during cell differentiation on Ch films, the activity of MMP2 and MMP9 was determined, at different time points, by gelatin zymography. The results of three independent experiments using different donors show that throughout monocyte differentiation to macrophages, the levels of the pro-form of MMP9 are higher on Ch films compared to control conditions (Fig. 5A). Moreover, at later differentiation stages, i.e. at days 7 and 10, differences were also observed for the active form of MMP9, which gradually increased (Fig. 5A). In terms of MMP2 activity, only one band, corresponding to the pro-form of the enzyme, could be observed and, in contrast with MMP9, no differences were detected with macrophage differentiation on both substrates (Fig. 5A). Furthermore, the activity of both MMP2 and MMP9 produced by DC does not seem to be influenced by culture on Ch films (Fig. 5B).





**Fig. 5.** Activity of MMPs from macrophages and DC cultured on Ch films. Supernatants from macrophages and DC cultured for different time periods on Ch or TCPS were analysed by gelatin zymography. A representative gel of three independent experiments for MMP9 and MMP2 secretion by macrophages (**A**) or DC (**B**) is depicted. Quantitative assessment of the two proteolytic enzymes is also illustrated for macrophages (A-lower panels) and for DC (B-right panel). Results are expressed as a percentage of MMP activity of TCPS differentiated cells. TCPS, tissue culture polystyrene; Ch, chitosan; MMP, matrix metalloprotease.

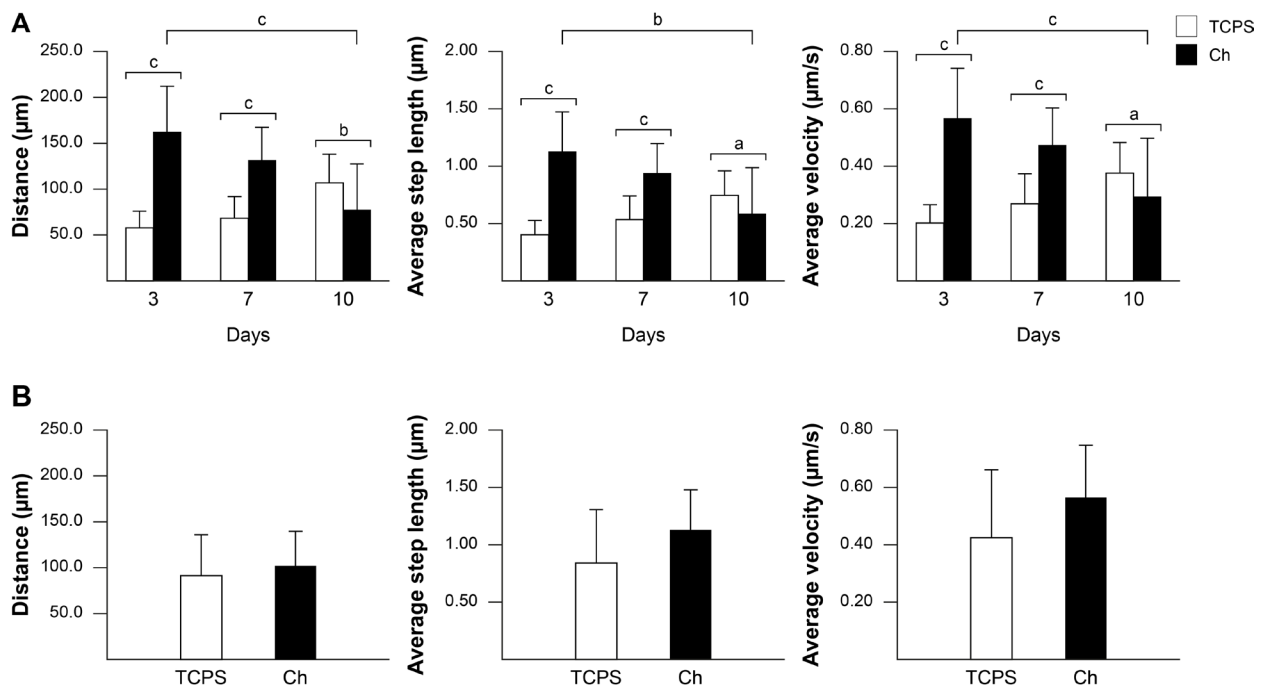
### Chitosan increases the migratory behaviour of macrophages without affecting DC migration

MMP9 activity has been previously described to assist the migration of monocytes and DC (Webster and Crowe, 2006), so we sought to investigate whether Ch has the potential to affect the migratory behaviour of these cell populations, by evaluating cell migration upon contact with Ch films. Thereby, cells at distinct stages of differentiation were monitored for 12 h by time-lapse video microscopy. The recorded images were merged to form a time-lapse sequence and trajectories analysed to determine the distance covered by each cell. Cell tracking indicates that at days 3 and 7 monocytes/macrophages have increased migratory behaviour, moving longer distances and at a higher speed on Ch than on TCPS control (Fig. 6A). In addition, the average step length, i.e. the average distance covered per frame, was also higher on Ch than on TCPS. However, with progression of differentiation macrophages show reduced migratory capacity, and when

fully differentiated, are slower and migrate significantly less on Ch films than on TCPS control ( $p < 0.05$ ) (Fig. 6A). Conversely, upon contact with the substrates, DC do not show any significant differences (Fig. 6B). Nevertheless, on Ch the distance, average step length and velocity were higher for differentiated DC (day 7) than for fully differentiated macrophages (day 10). In fact, the migratory behaviour of DC is similar to that of macrophages at day 7 of differentiation (Fig. 6).

### Ch-activated macrophages and DC do not promote significant T cell proliferation

Having established that Ch renders macrophages and DC active and affects their cytokine profile, it was important to address whether this biomaterial was able to trigger an APC-mediated T lymphocyte activation and consequently an adaptive immune response. The stimulatory capacity of Ch, *per se*, on lymphocytes was firstly evaluated. After 20 h of exposure to Ch films, the levels of the early



**Fig. 6.** Migratory behaviour of macrophages and DC on Ch. Human primary monocyte-derived macrophages differentiated for 3, 7 or 10 d or monocyte-derived DC cultured for 7 d in TCPS were transferred to chitosan films or new TCPS wells, and allowed to adhere for 2 h before being monitored for 12 h using time-lapse video microscopy. Each frame was merged to produce a time-lapse sequence and cells were tracked for the total distance travelled. Distance, average step length and average velocity of macrophages (**A**) or DC (**B**) were determined using FIJI software. Error bars represent standard deviation of, at least, 12 cells per condition and superscripts indicate statistically significant difference (<sup>a</sup> $p < 0.05$ ; <sup>b</sup> $p < 0.01$ ; <sup>c</sup> $p < 0.001$ ). TCPS, tissue culture polystyrene; Ch, chitosan.

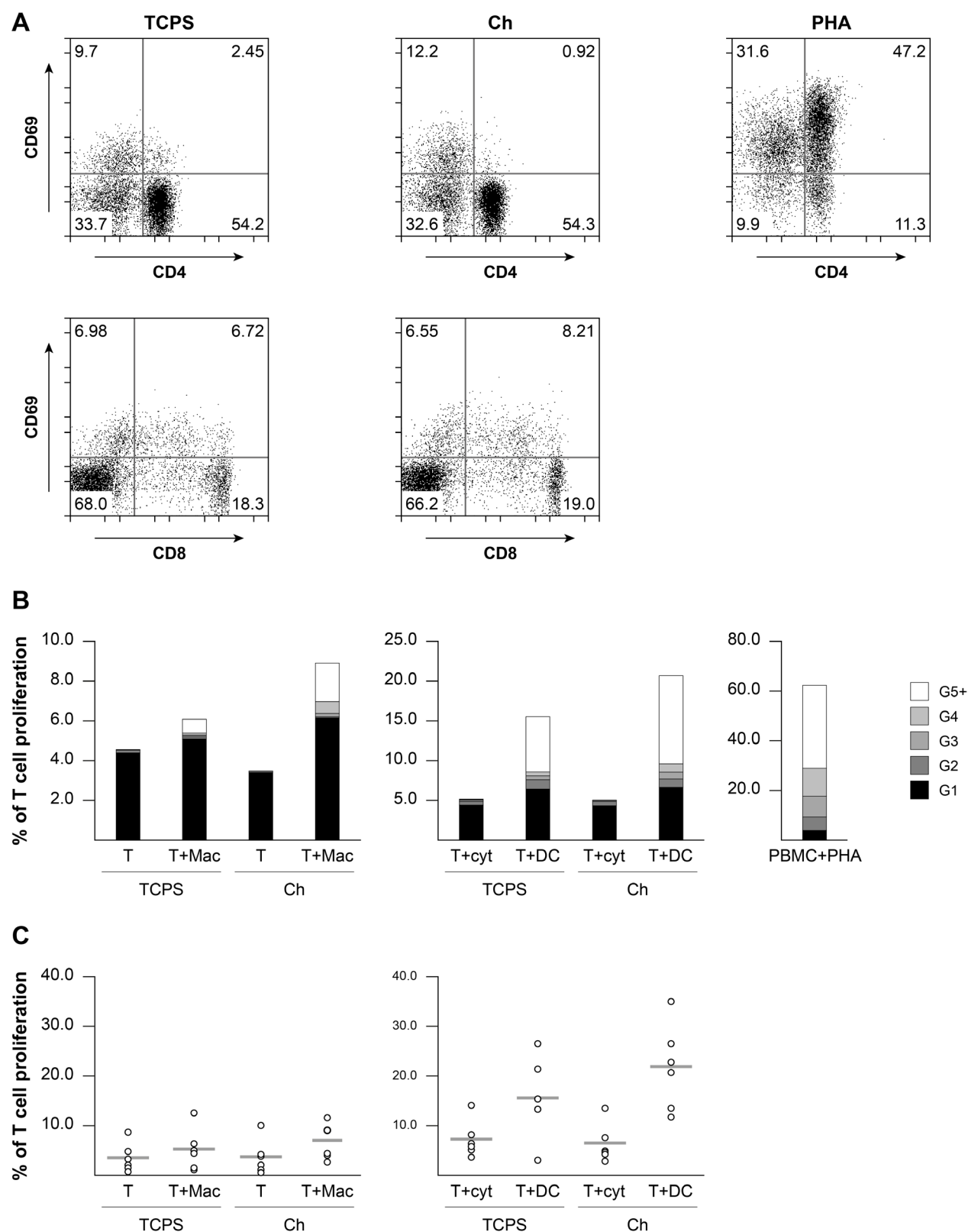
T lymphocyte activation marker CD69 were measured and found to be similar to TCPS control, with only very few CD8<sup>+</sup> T cells expressing CD69 (Fig. 7A). This is in clear contrast with PHA-stimulated lymphocytes used as positive control, where most T cells expressed CD69. To address Ch-induced macrophage and DC functionality, mixed lymphocyte reactions were set up and T lymphocyte proliferation evaluated. The results obtained show that the highest percentage of T lymphocyte proliferation occurred when these cells were in contact with DC, either on TCPS or Ch substrates (Fig. 7B). Notably, lymphocytes alone or with DC differentiating cytokines tend to divide just once, whereas a small percentage of those cultured with DC or macrophages divide many times (G5+). In Fig. 7C the percentage of T lymphocytes that divided, across six independent experiments, is presented. Despite the slightly higher T cell proliferation when either macrophages or DC were differentiated and co-cultured with T cells, on Ch as compared to TCPS, no significant differences were found (Fig. 7C). The levels of T cell proliferation observed in all conditions tested were well below the strong non-specific response induced by PHA (Fig. 7B).

### Discussion

In this study, for the first time, macrophages and DC were differentiated from the same precursor cells, directly on Ch

films for 10 or 7 d, respectively, in order to elucidate the influence of this polymer on cell behaviour. In contrast to other reports, cell-Ch interactions were not evaluated after a short exposure but during all the differentiation process and when Ch is presented as a substrate. In addition, despite various studies performed with macrophages, to the best of our knowledge none has addressed Ch-induced macrophage polarisation, very few involved dendritic cells and none made a comparison between the pro- and anti-inflammatory cytokines secreted by both. Absence of exogenous cytokines, other than those required for inducing DC differentiation from monocytes, assures that the observed responses result only from biomaterial stimulation and are not pre-conditioned. The data obtained supports and extends previous reports on Ch-induced macrophage and DC activation, but most of all indicates that Ch is capable of modulating macrophage polarisation towards an M2 phenotype, while driving a more pro-inflammatory DC differentiation. The responses elicited by Ch on macrophages and DC showed differences and similarities, although a more pronounced effect was found on macrophages.

When a device is implanted, monocytes – and not fully differentiated cells – are recruited and adhere to the implant surface, subsequently differentiating into macrophages (Anderson *et al.*, 2008) or potentially DC (León *et al.*, 2005). Interaction of both macrophages and DC with biomaterials *in vivo* has been demonstrated previously



**Fig. 7.** T cell activation and proliferation in response to Ch films or to antigen-presenting cells differentiated on distinct substrates. **(A)** Peripheral blood lymphocytes were cultured on Ch for 20 h, harvested and double stained for the early activation marker CD69 and CD4 or CD8 to identify specific T cell subsets. PHA was used as positive control. **(B, C)** To evaluate T cell proliferation, mixed lymphocyte reactions were set up on Ch and TCPS and T cell proliferation evaluated by CFSE halving of CD3<sup>+</sup> cells after 7 d of co-culture. Unpurified lymphocytes from the same donor as purified T cells were stimulated with PHA for control purposes. The percentage of T cells in each generation **(B)** and the percentage of T cells that divided at least once for each of 6 independent experiments **(C)** are depicted. TCPS, tissue culture polystyrene; Ch, chitosan; T, T lymphocytes; Mac, macrophages; DC, dendritic cells; cyt, cytokines; PHA, phytohaemmagglutinin; PBMC, peripheral blood mononuclear cells.

(Anderson *et al.*, 2008; Babensee, 2008) and is likely crucial for the development of a foreign body reaction. Therefore, to more closely mimic physiological conditions, studies involving the interaction between macrophages and biomaterials have been performed during 10–14 d of culture, directly on the substrate under investigation (Anderson *et al.*, 2008; Ariganello *et al.*, 2011; Dinnes *et al.*, 2008). Such an approach allows monitoring of cellular changes at different stages of differentiation, thus enabling a full characterisation of biomaterial-mediated effects. As for DC, 7 d are reported to be necessary to analyse their full differentiation and potential maturation in response to a given stimulus (Babensee and Paranjpe, 2005; Chapuis *et al.*, 1997). Thus, this time-point was chosen for analysing the effects of Ch on DC. In this study, the purity of the starting monocytic population was carefully considered, since low purity might have compromised the interpretation of specific cell-biomaterial interactions, given that cellular cross-talk between distinct cell populations has been reported to regulate each other's behaviour (Brodbeck *et al.*, 2005; Chang *et al.*, 2009). By using highly enriched monocytes we specifically addressed the ability of Ch to modulate macrophage and DC differentiation, with phenotypic and functional changes resulting only from substrate stimulation.

Ch is a natural biodegradable polymer with putative immunological properties, shown to enhance the function of different cell types, such as polymorphonuclear leukocytes, macrophages and fibroblasts (Mori *et al.*, 1997; Peluso *et al.*, 1994; Porporatto *et al.*, 2003; Ueno *et al.*, 2001b). It accelerates the infiltration of inflammatory cells *in vivo*, as well as the production of biological mediators, and also enhances wound healing (Barbosa *et al.*, 2010; Ueno *et al.*, 2001a; Ueno *et al.*, 2001c; VandeVord *et al.*, 2002). Upon implantation, Ch has been reported to have a high degree of biocompatibility in different conditions and to lead to an inflammatory reaction that depends on the DA of the polymer (Azab *et al.*, 2007; Kim *et al.*, 2011; VandeVord *et al.*, 2002; Yussof *et al.*, 2011). The DA of Ch used in this study (DA = 11 %) is below the range described in the literature as inducing marked inflammation (Barbosa *et al.*, 2010; Hidaka *et al.*, 1999; Tomihata and Ikada, 1997). In fact, Ch implants with the same DA did not induce prolonged or exacerbated inflammatory responses throughout the implantation period (Yussof *et al.*, 2011).

In this report, we present evidence that macrophages polarise into an M2c-like phenotype in response to Ch stimulation. This conclusion was based essentially on the combined analysis of cell surface markers and cytokine secretion profile. Down-regulation of cell surface MHCII and the co-stimulatory molecule CD86, was the first indication excluding an M1-pro-inflammatory polarisation (Mantovani *et al.*, 2004). Importantly, within the anti-inflammatory M2 phenotype, only the M2c subset includes cells devoid of MHCII at the cell surface (Kou and Babensee, 2011; Mantovani *et al.*, 2004). Similarly, the different levels of cell surface expression detected on Ch for CD206 and CD163, commonly used as M2 phenotypic markers, point to an M2c-like polarisation. In fact, of the three M2 subsets, both M2a and M2c express CD206 (Mantovani *et al.*, 2004), as do approximately

43% of CD14<sup>+</sup> Ch-differentiated macrophages. Moreover, our results agree with a previous report showing that the levels of CD206 are not affected by several pro- and anti-inflammatory stimuli, including LPS or IL-10 (Porcheray *et al.*, 2005), and this appears also to be the case for Ch. The discrepancy between our data and that of Ueno *et al.* (2001a), who demonstrated that murine peritoneal macrophages up-regulate the expression of CD206 after being treated for 6 h with Ch-supplemented media, may not only be species-driven, but simply lie on the time and way the stimulating agent was presented to the cells. Since the N-acetyl-glucosamine residues of Ch can interact with CD206, it is conceivable that in a short-term interaction, macrophages recognise Ch via CD206, thereby increasing its expression at the surface (Han *et al.*, 2005). Nonetheless, after 10 d of culture on Ch, expression and activity of this receptor may be differently modulated by divergent mechanisms, possibly important during macrophage differentiation and cellular activation (Fiani *et al.*, 1998). Regarding CD163, its increased expression is more associated with the M2a class and not with M2b or M2c (Mantovani *et al.*, 2004). The reduced levels of CD163 found on Ch could also result from increased secretion of TGF- $\beta$ 1 promoted by this substrate, that we and others (Ueno *et al.*, 2001c) observe, since TGF- $\beta$  acts as a negative regulator of CD163 expression by macrophages (Pioli *et al.*, 2004). Although many authors have reported and still consider up-regulation of CD163 following IL-10 treatment as indicative of an M2 phenotype, this is frequently associated with the addition of M-CSF to promote macrophage differentiation, as this cytokine has been shown to enhance CD163 expression (Porcheray *et al.*, 2005). In this study no differences were found between control conditions and IL-10 stimulation, probably because no exogenous cytokines were used to induce macrophage differentiation. Notably, despite the down-regulation of all phenotypic expression markers investigated, macrophages remained viable (as shown by Annexin and PI staining) and exhibited normal morphology during the entire culture period (data not shown), indicating that the cell surface marker profile observed is a specific Ch-induced effect, and is not related to a loss of cell viability.

Besides membrane receptors, macrophage polarisation is associated with other distinctive gene expression signatures, which include soluble factors and effector molecules (Martinez *et al.*, 2008). The levels of three pro-inflammatory cytokines, IL-6, TNF- $\alpha$  and IL-1 $\beta$ , on Ch substrates progressively decreased over time, whereas anti-inflammatory IL-10 concentration increased up to day 10. When the early differentiation time of 3 d was compared with 10 d of culture on Ch, a statistically significant decrease of TNF- $\alpha$  ( $p < 0.05$ ) and increase of IL-10 ( $p < 0.001$ ) secretion was observed. This gradual increase of IL-10 may explain the reduced levels of pro-inflammatory counterparts at later stages of macrophage differentiation, since IL-10 inhibits the production of several soluble mediators, including IL-6, TNF- $\alpha$  and IL-1 $\beta$  (Gordon, 2003). Regarding the pro-inflammatory cytokines IL-12 and IL-23, secretion levels measured on Ch were always low, which is a characteristic of M2 macrophages. Most importantly, macrophages cultured on Ch were found



to significantly increase the levels of the anti-inflammatory cytokine TGF- $\beta$ 1, described to be produced at elevated levels by M2c macrophages (Mantovani *et al.*, 2004). To the best of our knowledge there is no available data concerning cytokine profiles elicited by Ch scaffolds or Ch-coated implants. Nonetheless, our results concur with what has been previously observed for other forms of Ch *in vivo*. When given orally, Ch up-regulated IL-4 and TGF- $\beta$  mRNA expression and augmented IL-10 release at the mucosal levels (Porporatto *et al.*, 2005). Moreover, macrophage expression of IL-1, TNF- $\alpha$  and INF- $\gamma$  at the mRNA and protein levels are promoted by Ch, both *in vitro* and *in vivo* after a short-term stimulation (Feng *et al.*, 2004; Guzmán-Morales *et al.*, 2011; Nishimura *et al.*, 1986; Wei *et al.*, 2009). In this study, we also found an increase of pro-inflammatory cytokine production triggered by Ch at early monocyte to macrophage differentiation (day 3 of culture). Still, our time-course investigation revealed that progression of macrophage differentiation correlated with a decrease in pro-inflammatory cytokines, suggesting an M2-like macrophage polarisation. Previous reports have also demonstrated that *in vitro* monocyte/macrophage cytokine profiles may vary with biomaterial surface chemistry (Ariganello *et al.*, 2011; Brodbeck *et al.*, 2002; Gretzer *et al.*, 2003; Schutte *et al.*, 2009a). Of particular relevance to our studies were the observations of Jones and co-workers, who have reported a phenotypic switch of biomaterial-adherent macrophages over time to a phenotype that is close to that of alternatively activated macrophages, with down-regulation of IL-1 $\beta$  and IL-6, among others, and concomitant production of IL-10 (Jones *et al.*, 2007). Interestingly, a quantitative *in vivo* cytokine analysis showed a decrease of IL-1 $\beta$  together with an increase of IL-10 and TGF- $\beta$  over the implantation period using different synthetic biomedical polymers (Rodriguez *et al.*, 2009). Nevertheless, IL-6 and TNF- $\alpha$  levels varied with time, and with the polymer used (Rodriguez *et al.*, 2009). This indicates that distinct material-dependent cytokine profiles are associated with macrophages and foreign body giant cells *in vivo*. Likewise, evaluation of cytokine responses systemically (Walschus *et al.*, 2012) and from exudate fluids surrounding chemically distinct materials (Brodbeck *et al.*, 2003; Schutte *et al.*, 2009b) evidenced material-specific differences, particularly on IL-6, IL-10 and IL-13. Noteworthy, monocyte/macrophages purified from the foreign body reaction 3 d post-implantation up-regulated IL-1 $\beta$ , IL-6, IL-10 and TNF- $\alpha$  gene expression (Mesure *et al.*, 2010). Lastly, recent *in vivo* analysis of cytokine expression around implanted materials revealed the presence of IL-4, IL-13 and IL-10 in close proximity to the implant, which together with IL-6 and TGF- $\beta$ , co-localised with small macrophages (Higgins *et al.*, 2009).

To further confirm the M2c macrophage polarisation on Ch, supported by the cell surface marker expression pattern and cytokine secretion profile, production and activity of MMPs, which are described as being higher in M2 macrophages (Lolmede *et al.*, 2009), were analysed by gelatin zymography. Ch enhanced the activity of MMP9 produced by macrophages with time, without affecting that of MMP2. This reveals the specificity of this polymer in modulating macrophage MMP9 activity and corroborates

previous studies reporting the production of MMPs in a biomaterial-dependent manner (Jones *et al.*, 2008). There was some MMP9 production by TCPS-differentiated macrophages, as expected, because cell adherence has been shown to induce significant levels of this enzyme (Webster and Crowe, 2006). MMPs are involved in distinct processes, including regulation of cytokine activity and shedding of cell surface molecules (Webster and Crowe, 2006), such as CD163 and CD206 (Fabrick *et al.*, 2007; Martínez-Pomares *et al.*, 1998). Also, MMP9 has been suggested to participate in phagocytosis and degradation of Ch-DNA nanoparticles (Webster and Crowe, 2006). Nonetheless, a correlation between increasing MMP9 activity levels found on Ch and its degradation are beyond the scope of this study. However, as this enzyme also contributes to extracellular matrix remodelling and facilitates cellular migration, the migratory behaviour of monocytes/macrophages and DC placed in contact with Ch, at distinct differentiation stages, was evaluated. Cell motility analysis was the only assay in this study where cells were placed on Ch films upon differentiation. This was performed to allow us to assess substrate modulation of early migratory events taking place immediately after exposure of macrophage or DC to Ch. Moreover, it avoids possible interference in migration caused by previous cell adherence. Our results showed that the overall migratory ability of macrophages on Ch films decreases with their phenotypic progression, suggesting enhanced cell adhesion over time. This may result from alterations to specialised adhesion structures, such as podosomes, which have been shown to occur during macrophage differentiation (DeFife *et al.*, 1999) and to be biomaterial-dependent for both macrophages and DC (Rogers and Babensee, 2011).

Contrary to macrophages, supernatants of DC differentiated on Ch films *versus* TCPS did not show differences in MMP9 activity. This agrees with the similar migratory ability of DC cultured on Ch films as compared to control surfaces, as increased MMP9 is known to enhance DC migration (Webster and Crowe, 2006). Regarding DC cytokine profile, Ch elicited significant increases of both TNF- $\alpha$  and IL-1 $\beta$ , in comparison to cells cultured on TCPS, a different response to that observed for fully differentiated macrophages. The morphology of DC cultured on Ch revealed that the polymer activates these cells (data not shown), which, together with a significant reduction of IL-10 secretion and up-regulation of the co-stimulatory molecule CD86, point towards a pro-inflammatory response by DC. However, this appears not to be a classical pro-inflammatory response, as levels of IL-12 and IL-23, two main pro-inflammatory cytokines, remained low and levels of TGF- $\beta$ 1 increased. These events may be inter-related, since TGF- $\beta$  induces down-regulation of IL-12 (Fainaru *et al.*, 2007), which shares a subunit with IL-23 (Oppmann *et al.*, 2000). Moreover, the similar expression levels found for the activation marker CD83 and MHCII molecule HLA-DR on Ch and TCPS, as previously reported (Babensee and Paranjpe, 2005), also support this assumption.

In this study, we also investigated the action of Ch on T cells, which were used to assess DC and macrophage functional characteristics. T cells, by themselves,

were poorly triggered by Ch, as demonstrated by the scarce expression of CD69 and low proliferation when cultured alone on Ch films. As expected, a small T cell population responds to allogeneic macrophages or DC by proliferating. However, when several pairs of donors were analysed, no significant differences were found between the proliferation rates of T cells stimulated by DC or macrophages differentiated on Ch substrates, in relation to the basal proliferation rates induced by unstimulated cells, differentiated just on TCPS. Thus, despite some level of macrophage and DC activation on Ch, this does not seem to translate into the induction of an adaptive immune response, characterised by increased T lymphocyte proliferation. Notably however, this moderate level of DC maturation supported by Ch possibly explains the observed adjuvant effect of the polymer in the immune response towards associated antigens (Slütter *et al.*, 2010), a strategy currently used for vaccine delivery using polymers like Ch (Arca *et al.*, 2009; Kang *et al.*, 2009). Although DC have been shown *in vivo* to transport Ch to lymph nodes when the polymer is administered orally (Porporatto *et al.*, 2005), and to be present at the implant site when PLGA scaffolds were used (Babensee, 2008), further experiments are necessary to show DC interaction with Ch scaffolds *in vivo*. In such a scenario, Ch may be able to influence the immune response to biological components of allogenic or xenogenic origin incorporated in the biomaterial, by acting as an adjuvant capable of inducing immunity (Arca *et al.*, 2009; Kang *et al.*, 2009; van der Lubben *et al.*, 2001), or promoting tolerance, as reported by Porporatto and co-workers (Porporatto *et al.*, 2009). In the case of this study, we observe a slight activation of DC without significant induction of T cell proliferation, together with a macrophage phenotypic switch over time and significant amounts of TGF- $\beta$ 1 produced by both cell populations. This is likely to result in a more anti-inflammatory environment, promoting wound healing and tissue repair, towards enhanced integration of the biomaterial, as previously described (Kim *et al.*, 2011; VandeVord *et al.*, 2002; Yussuf *et al.*, 2011).

Altogether, the findings presented herein suggest that Ch affects differently macrophage and DC differentiation and modulates macrophage polarisation. As M1 and M2 macrophages represent extremes along a continuum that includes multiple phenotypes (Mantovani *et al.*, 2004), one can suggest that the position assumed by Ch-differentiated macrophages in this spectrum would be close to the M2c phenotype. As for DC differentiated on Ch films, they seem to adopt a more pro-inflammatory phenotype when challenged by Ch. Based on all the results obtained we hypothesise that an overall pro-inflammatory reaction associated to a particular biomaterial may sometimes hide the expression of anti-inflammatory cytokines, which may be of relevance for tissue repair/regeneration. In addition, combined secretion of the strong pleiotropic mediator TGF- $\beta$ 1 from both cell types upon Ch stimulation, suggests that Ch should have some inherent, although perhaps limited, ability to support tissue repair/regeneration. Control of macrophage and DC phenotype from pro-inflammatory to regulatory/tolerogenic is of much interest for tissue engineering applications. Subsequent

modification of this polymer with appropriate agents may further enhance Ch ability to modulate cell polarisation and constitute a strategy for faster and improved wound-healing and tissue repair.

## Conclusion

This work analysed the influence of Ch, as a substrate, on the differentiation of two distinct cell populations with a common precursor: macrophages and monocyte-derived DC. By allowing cellular differentiation to occur directly on Ch, it was found that, with time, this polymer induced a phenotypic switch on macrophages to an activation state similar to an M2c polarisation. In addition, Ch elicited DC pro-inflammatory activation without leading to significant T cell proliferation. Taken together, our results indicate different outcomes for the related cell populations studied, with likely implications for the host response following implantation. These data highlight the need for dissecting the effects of biomaterials on different inflammatory cell populations, which is of particular relevance in the design of biomaterials capable of stimulating tissue repair/regeneration through proper tuning of pro- and anti-inflammatory responses.

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## Discussion with Reviewers

**Reviewer I:** How do results presented in this paper complement or explain *in vivo* data collected for inflammatory or host reactions to chitosan?

**Authors:** Our results, which indicate an anti-inflammatory polarisation of macrophages and moderate pro-inflammatory DC stimulation, may provide an explanation for the high biocompatibility of chitosan (Ch) found *in vivo* in different tissues and for diverse applications. Although showing recruitment of inflammatory cells,

Ch was also demonstrated to induce low inflammatory reactions (Azab *et al.*, 2007; Kim *et al.*, 2011; VandeVord *et al.*, 2002; Yussof *et al.*, 2011). Even though some studies have documented exacerbated inflammatory reaction to Ch (Crompton *et al.*, 2006; Hidaka *et al.*, 1999; Peluso *et al.*, 1994), it has also been shown that it is dependent on the degree of acetylation (DA) of the polymer (Barbosa *et al.*, 2010; Hidaka *et al.*, 1999; Tomihata and Ikada, 1997). In our case, the DA of the Ch used was below the range described to trigger severe inflammation (Hidaka *et al.*, 1999) and Ch implants with the same DA did not induce any prolonged or exacerbated inflammatory responses throughout the implantation period (Yussof *et al.*, 2011). Of note, most of the *in vivo* studies on the inflammatory reaction to Ch looked at number of recruited cells and their lineage and not at their activation or polarisation status (Barbosa *et al.*, 2010; Crompton *et al.*, 2006; Hidaka *et al.*, 1999; Peluso *et al.*, 1994; Tomihata and Ikada, 1997; Yussof *et al.*, 2011). Finally, Ch has been reported as an adjuvant in immunotherapies (Muzzarelli, 2010).

**Reviewer I:** What is the *in vivo* evidence that DCs (distinct from macrophages) actually elicit and are involved in biomaterial-based (and specifically chitosan) inflammatory modulation?

**Authors:** The presence of dendritic cells at the implant site *in vivo* has been demonstrated using PLGA scaffolds, either in the presence or absence of an antigen (reviewed in Babensee, 2008). As for Ch in particular, it has also been demonstrated *in vivo* that orally administered Ch is taken by DC to the draining lymph nodes, in the case of Peyer's patches (Porporatto *et al.*, 2005), and to enhance tolerance to an articular antigen (type II collagen) with a decrease in the inflammatory responses (Porporatto *et al.*, 2009).

**Reviewer I:** The work by Leon *et al.* (2005) describes general evidence of *in vitro/in vivo* monocyte to DC differentiation, but does not provide any proof of DC involvement in a chitosan foreign body response. What is known about cellular components of foreign body response to chitosan implants?

**Authors:** To the best of our knowledge no study has investigated or identified yet the presence of DC in Ch scaffolds. However, DC recruitment to the implant site and interaction with PLGA scaffolds after 7 d post-implantation have been reported (Babensee, 2008). As for the cellular components of foreign body response to Ch implants, VandeVord and co-workers noted a dramatic infiltration of neutrophils, which were not activated, and that resolved with increasing implantation time (VandeVord *et al.*, 2002). Four weeks post-implantation, a population with fibroblastic morphology was noticed. At later implantation stages, a variety of cells were observed within the Ch scaffold, including monocytes, macrophages, and giant cells, although the vast majority appeared to be fibroblasts (VandeVord *et al.*, 2002). Similarly, Peluso *et al.* (1994) also reported the presence of many leukocytes at the polymer-tissue interface 14 d post-implantation. In addition, a mild foreign body response to Ch, in comparison with absorbable surgical sutures (Azab *et al.*, 2007), and to Gore-Tex and Vicryl (Kim *et al.*, 2011) has been

reported. Azab and co-workers found polymorphonuclear cells surrounding the implant at day 1 post-implantation, occasional macrophages at day 3, and a decrease of the inflammatory infiltrate by day 14 (Azab *et al.*, 2007). Finally, no adverse foreign body reaction to a Ch bilayer as compared to two commercially available skin substitutes was found, with the Ch group showing only low levels of acute inflammatory cells (Yussof *et al.*, 2011).

**Reviewer II:** What is the mechanism or how could this different response to chitosan be explained.

**Authors:** The response of macrophages and DC to Ch is different but shows converging aspects, namely the production of TGF- $\beta$  and the lack of T cell stimulation. DC display a more pro-inflammatory phenotype than macrophages, yet their response does not correspond to a full-blown inflammatory reaction. For example, an increase of the co-stimulatory molecule CD86 is not accompanied

by increasing expression of the activation marker CD83 or MHC-class II (HLA-DR), and there is no significant T cell stimulation. Also, in terms of cytokines, although we observe increased production of pro-inflammatory TNF- $\alpha$  and IL-1 $\beta$  combined with a reduction of anti-inflammatory IL-10, we do not see increased IL-12, a major hallmark of inflammation. We also observe an increase of TGF- $\beta$ . Thus, we believe that in the presence of Ch alone, both macrophages and DC are likely to contribute to a pro-regenerative environment.

#### Additional Reference

Muzzarelli RA (2010) Chitins and chitosans as immunoadjuvants and non-allergenic drug carriers. *Mar Drugs* **8**: 292-312.