

Butyrate affects differentiation, maturation and function of human monocyte-derived dendritic cells and macrophages

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SUMMARY

We studied the *in vitro* effects of butyric acid on differentiation, maturation and function of dendritic cells (DC) and macrophages (M Φ) generated from human monocytes. A non-toxic dose of butyrate was shown to alter the phenotypic differentiation process of DC as assessed by a persistence of CD14, and a decreased CD54, CD86 and HLA class II expression. The more immature differentiation stage of treated cells was confirmed further by their increased phagocytic capability, their altered capacity to produce IL-10 and IL-12, and their weak allostimulatory abilities. Butyrate also altered DC terminal maturation, regardless of the maturation inducer, as demonstrated by a strong down-regulation of CD83, a decreased expression of CD40, CD86 and HLA class II. Similarly, butyrate altered M Φ differentiation, down-regulating the expression of the restricted membrane antigens and reducing the phagocytic capacity of treated cells. To investigate further the mechanism by which butyrate hampers the monocyte dual differentiation pathway, we studied the effects of 1,25(OH)₂D₃ alone or in combination with butyrate on the phenotypic features of DC. Unlike 1,25(OH)₂D₃, butyrate inhibited DC differentiation without redirecting it towards M Φ . Combined treatment gave rise to a new cell subset (CD14^{high}, CD86 and HLA-DR^{low}) phenotypically distinct from monocytes. These results reveal an alternative mechanism of inhibition of DC and M Φ differentiation. Altogether, our data demonstrate a novel immune suppression property of butyrate that may modulate both inflammatory and immune responses and support further the interest for butyrate and its derivatives as new immunotherapeutic agents.

Keywords butyrate dendritic cells immunosuppression macrophages monocytes

INTRODUCTION

A novel form of immune intervention mediated by the G1 blocker butyric acid has been proposed recently for the tolerance induction to allografts [1,2] and the treatment of autoimmune diseases [3]. Butyrate and other short-chain fatty acids are end-products of fibre polysaccharide metabolism in the intestine and affect key functions of the colonic epithelium *in vivo* [4]. Butyrate has been studied primarily as an antineoplastic agent able to inhibit proliferation and to induce differentiation in a variety of transformed cell types *in vitro* (for a review, see [5]). In addition to its effects on tumour cells, butyrate has also been shown to possess anti-inflammatory properties [6–8] and to inhibit T cell activation in response to mitogens [9,10], alloantigens [11] and soluble antigens [12]. Modulation of proliferative T cell responses

was reported to result from both direct interference with cell cycle progression [13] and alteration of the stimulatory function of antigen presenting cells (APC) [14]. Based on the observation that pretreatment of monocytes with butyrate suppresses subsequent T cell proliferation responses and down-regulates expression of B7-1 (CD80), ICAM-1 (CD54) and LFA-3 (CD58) on monocytes, it has been speculated that altered APC function by butyrate was a consequence of their inability to deliver critical costimulatory signals [14]. However, these effects were observed only with high doses of butyrate which resulted in alteration of membrane integrity owing to apoptotic cell death [14] and, consequently, cell toxicity may represent the critical mechanism underlying altered APC function in the monocyte model.

Important clues to understand the anti-APC action of butyrate might come from the analysis of the cytodifferentiation capabilities of this agent on monocyte-derived cells. Indeed, peripheral blood monocytes can differentiate *in vivo* into both macrophages (M Φ) and dendritic cells (DC), depending upon their cytokine environment [15]. This indicates a dichotomy of

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monocyte differentiation either into antigen-presenting DC leading to specific immunity or into phagocytosing M Φ , thus connecting non-specific and specific immune responses [16]. Over the past years, methods have been described to differentiate M Φ [17,18] and DC [19,20] from blood monocytes by *in vitro* culture with granulocyte-macrophage colony-stimulating factor (GM-CSF) or GM-CSF plus IL-4, respectively. In addition cultured DC, which show functional and phenotypic characteristics typical of the immature stage of differentiation, can be differentiated further *in vitro* into mature DC in the presence of tumour necrosis factor- α (TNF- α) and prostaglandin E₂ (PGE₂) [21]. PGE₂ is a mediator found in inflamed tissues and secreted by tumours (reviewed in [22]). Therefore, using such an *in vitro* cultured cell model, the objective of the present study was to investigate whether butyrate affects differentiation, maturation and function of monocyte-derived M Φ and DC, thus contributing to the anti-inflammatory and immunosuppressive properties of this molecule.

MATERIALS AND METHODS

Cytokines and reagents

All culture media, chemicals and reagents were purchased from GIBCO-BRL (Cergy-Pontoise, France) unless specified otherwise. Recombinant human GM-CSF was obtained from Schering Plough (Levallois Perret, France), recombinant human IL-1 beta (IL-1 β) and IL-4, TNF- α , γ -interferon (IFN- γ) from R&D Systems (Abingdon, UK). PGE₂, indomethacin, LPS, butyrate and 1,25(OH)₂D₃ were obtained from Sigma (L'Isle d'Abeau Chesnes, France), *Staphylococcus aureus* cowan 1 strain (SAC) from Calbiochem (Meudon, France) and human pooled AB serum from Valbiotech (Paris, France).

Cell separation

After giving informed consent, healthy blood donors underwent mononuclear cell leukapheresis using a continuous-flow cell separator (Spectra, COBE laboratory, Lakewood, CO, USA) and ACD-A anticoagulant (Maco Pharma, Tourcoing, France). Purification of both monocytes and lymphocytes was performed by countercurrent centrifugal elutriation using the J6 MC centrifuge equipped with the JE 5.0 rotor (Beckman, Roissy, France). Briefly, the rotor was sterilized by autoclaving (20 min at 120°C) and primed by a solution supplemented with 1% human albumin (Bruneau, Boulogne-Billancourt, France). The initial cell loading flow rate was set to 40 ml/min (initial rotor speed, 2000 r.p.m. at 4°C). Elutriation was performed by stepwise increments of the flow rate. The size and concentration of elutriated cells were closely monitored by flow cytometry using an EPICS XL flow-cytometer (Coulter, Villepinte, France). At a flow rate of 45–55 ml/min, lymphocyte-rich fraction was collected. The flow was slowed progressively to 30 ml/min to reduce the dilution of the elutriated sample. Monocyte-rich fraction was elutriated by progressively decreasing the rotor speed to 1400 r.p.m. and collected on ice. Cell viability assessed by the trypan blue exclusion test was always >98% for both lymphocytes and monocytes. T lymphocyte and monocyte purity as determined by CD2 and CD14 expression, respectively, was always >90%.

Human DC and M Φ cultures

DC and M Φ were generated from purified monocytes in Teflon bags under adherence-free conditions as described previously [23,24]. Briefly, M Φ were differentiated in IMDM supplemented

with 2 mM L-glutamine, 1% non-essential amino acids, 100 mM sodium pyruvate, 3×10^{-5} M 2-mercaptoethanol, 5% human pooled AB serum, 5×10^{-6} M indomethacin and 250 U/ml GM-CSF, in the presence or absence of butyrate. Similarly, immature DC were differentiated from monocytes cultured in RPMI-1640 supplemented with 2 mM L-glutamine, 5% human pooled AB serum, 400 U/ml GM-CSF and 500 U/ml IL-4, in the presence or absence of butyrate. In some experiments various concentrations of 1,25(OH)₂D₃ ranging from 10^{-10} M to 10^{-7} M were added concomitantly to butyrate.

DC maturation was induced by adding 1000 U/ml TNF- α and 3 μ g/ml PGE₂, on 5-day cultured DC, in the presence or absence of butyrate. Cells were recovered after 48 h of culture. Some experiments were performed using 1000 U/ml TNF- α plus 15 μ g/ml IL-1 β , or 1 μ g/ml LPS as maturation inducers.

Cell immunophenotyping

Cell staining was performed using the following mouse monoclonal antibodies, all from Immunotech (Marseille, France) except when indicated: anti-CD14 PE (Dako, Trappes, France), anti-CD16 FITC, anti-CD40 PE, anti-CD54 PE, anti-CD80 FITC, anti-CD83 PE, anti-CD86 PE (Becton Dickinson, Pont-de-Claix, France), anti-HLA-DR FITC (Becton Dickinson, Pont-de-Claix, France) and anti-HLA-DQ FITC.

IL-10 and IL-12 production

Activated DC were assessed for their cytokine production according to the method described by Hilkens *et al.* [25]. Briefly, 1×10^6 cells were stimulated in 12-well flat-bottom culture plates in serum-free AIM-V medium containing 1% pyruvate in a final volume of 1 ml. SAC strain (75 μ g/ml) or SAC strain (75 μ g/ml) plus 1000 UI/ml IFN- γ were used to induce either IL-10 or IL-12 secretion. After 24 h, supernatants were collected and cytokine production was determined by enzyme-linked immunosorbent assay (ELISA) using commercial kits purchased from R&D Systems (Abingdon, UK) according to the manufacturer's instructions. The detection limit of this ELISA was <0.5 pg/ml for IL-10 and IL-12.

Antigen capture

Single cell phagocytosis quantification was assessed according to the method adapted from Dunn and Tyrer [26]. A suspension of 2 μ m FITC-dextran microbeads (Polysciences, Warrington, PA, USA) was added in Teflon bags. After a 4-h incubation period at 37°C, cells were recovered and washed extensively with cold Dulbecco's PBS to stop phagocytosis. Then, cells were resuspended in PBS at a final concentration of 5×10^5 cells/ml and analysed by flow cytometry. In this assay, cell fluorescence intensity depends on the number of beads that have been phagocytized. Therefore, when the number of cells is analysed as a function of the fluorescence intensity, each peak depicted clusters of cells that have phagocytized 1, 2, 3 or more microbeads.

Allogeneic mixed leucocyte reaction (MLR)

The stimulation of purified T lymphocytes by immature or mature DC cultured in the presence or absence of butyrate was evaluated in the MLR assay as described elsewhere [27]. Briefly, stimulator cells were added in graded doses to 2×10^5 allogeneic responder T cells in 96-well flat-bottomed tissue culture plates (0.2 ml of medium per well). Cell proliferation during the last 18 h was quantified by incubation of cells with 1 μ Ci (37 kBq) of

[³H]-thymidine (Amersham, Les Ulis, France). The cells were harvested onto filters and radioactivity was measured in a scintillation counter.

Statistical analysis

Statistical analysis was performed using StatView IV software (Abacus Concepts INC, Berkeley, CA, USA). Results are expressed as mean \pm s.e.m. Comparisons were performed using analysis of variance followed by Fisher's exact test when appropriate. A *P*-value ≤ 0.05 was considered statistically significant.

RESULTS

Phenotypic characterization of monocyte-derived cells used in this study

Flow cytometric analyses were performed to confirm phenotypic differences between monocytes, DC and M Φ in our culture system. DC and M Φ were generated from monocytes cultured for 7 days in the presence of either GM-CSF plus IL-4 or GM-CSF alone, respectively, according to the method described previously [24]. As shown in Fig. 1a, the DC population expressed the typical phenotype of immature DC [19,20,24], with low CD80, CD86 and no CD83, whereas M Φ grown in parallel showed a different pattern of surface marker expression, as assessed by increased CD14 and CD16 expression. We next investigated the phenotypic changes in immature DC exposed to the cocktail TNF- α plus PGE₂ during the last 2 days of their culture. Cultured cells exhibited a phenotype commonly referred to as mature DC [21], characterized by increased CD83, CD86 and HLA class II molecule expression (Fig. 1b).

Butyrate alters the phenotypic differentiation of monocyte-derived immature DC and M Φ

To examine the toxicity of butyrate in our culture system, dose-response curves, ranging from 0.1 to 10 mM, were carried out based on cell recovery and viability. As calculated from five independent experiments, induction of cell death was dose-dependent with a decrease in DC yields from $94 \pm 4\%$ (mean percentage \pm s.e.m.) in the presence of 0.5 mM butyrate to $52 \pm 2\%$ in the presence of 10 mM butyrate. In the same way, M Φ yields decreased from $91 \pm 5\%$ to $47 \pm 6\%$ in the presence of 0.5 mM and 10 mM butyrate, respectively. Therefore, a standard butyrate concentration of 0.5 mM was chosen for subsequent experiments.

Phenotypic characteristics of immature DC and M Φ cultured in the presence or absence of butyrate were analysed using flow cytometry and compared to freshly isolated monocytes from the same individual (Fig. 2). Untreated DC down-regulated CD14, neo-expressed CD40, CD54, CD80, CD86 and up-regulated the expression of MHC class II products HLA-DR and HLA-DQ. As shown in Fig. 2a, treatment by butyrate partially inhibited this phenotypic differentiation process and resulted in a maintained CD14 expression and in a limited increase in CD40, CD54, CD86 and MHC II molecule expression. Similarly, the main phenotypic features acquired by M Φ during culture (i.e. up-regulated CD14, CD16, CD54, CD86 and MHC II molecules) were restrained by butyrate (Fig. 2b).

Butyrate alters the terminal maturation of DC

Upon activation by various stimuli, DC undergo terminal maturation associated with the modulation of relevant surface antigens [28]. To study the effect of butyrate on this maturation process,

Table 1. Effect of butyrate on the terminal maturation of DC. Day 5 DC were matured with TNF- α plus PGE₂ or TNF- α plus IL-1 β or LPS in the absence or in the presence of 0.5 mM butyrate. On day 7, cells were harvested and analysed by flow cytometry. Data represent the inhibitory effect of butyrate on CD83, CD86 and HLA-DR and are mean \pm s.e.m. inhibition percentage of three separate experiments performed with three different donors

Membrane markers	Maturation inducers		
	TNF- α plus PGE ₂	TNF- α plus IL-1 β	LPS
CD83	49.4 \pm 1.2%	32.6 \pm 0.9%	54.4 \pm 2.4%
CD86	26.3 \pm 1.9%	35.4 \pm 1.8%	32.3 \pm 1.5%
HLA-DR	50.2 \pm 2.3%	45.9 \pm 3.1%	48.6 \pm 2.1%

immature DC obtained after 5 days of standard culture were incubated for 2 additional days with TNF- α plus PGE₂ in the presence or absence of butyrate, and membrane phenotypes of mature cells were compared. As depicted in Fig. 3, butyrate strongly interfered with the DC maturation process by inhibiting the expression of the CD83 maturation marker and by reducing the cytokine-driven up-regulation of CD40, CD54, CD86 and MHC II molecules. Similar inhibition was observed when DC were matured using LPS or TNF- α plus IL-1 β (Table 1).

Butyrate modulates cytokine production by DC

We further investigated the capacity of butyrate to interfere with DC cytokine production. Compared to monocytes, control DC differentiated in the absence of butyrate exhibited a 20-fold decreased IL-10 secretion. In contrast, the presence of butyrate prevented this decrease partially and led to DC that exhibited an intermediate capacity to produce IL-10 (Fig. 4a). Kinetics of IL-12 secretion by DC cultured in the absence of butyrate (Fig. 4b) was characterized by a 10-fold increase on day 2 when compared to monocytes, followed by a drastic decrease. DC differentiated for 7 days in the presence of butyrate exhibited a preserved IL-12 secretion capacity similar to that observed on day 3 with untreated cultured cells (Fig. 4b). Both IL-10 and IL-12 data are consistent with the phenotypic and functional changes described above and confirm that butyrate hampers monocyte differentiation into DC.

Butyrate modulates antigen capture ability of immature DC and M Φ

Phagocytic capacity of DC and M Φ grown in the presence or absence of butyrate were analysed and compared to fresh monocytes. In a typical experiment shown in Fig. 5, 51% of immature DC are phagocytic (i.e. engulfed at least one microsphere) compared to 72% of monocytes. When focusing on cells expressing a high phagocytic capacity (more than 3 microspheres engulfed), the proportion decreased to 32% of DC versus 59% of monocytes. Altogether, these data suggest that monocyte differentiation into DC resulted in down-regulated phagocytic activity. The presence of butyrate inhibited this process totally, because 74% and 60% of treated cells engulfed at least 1 and more than 3 microspheres, respectively (Fig. 5b). Conversely, phagocytosis activity of monocytes was up-regulated upon differentiation into M Φ , with 80% and 67% of M Φ which engulfed at least 1 and more than 3

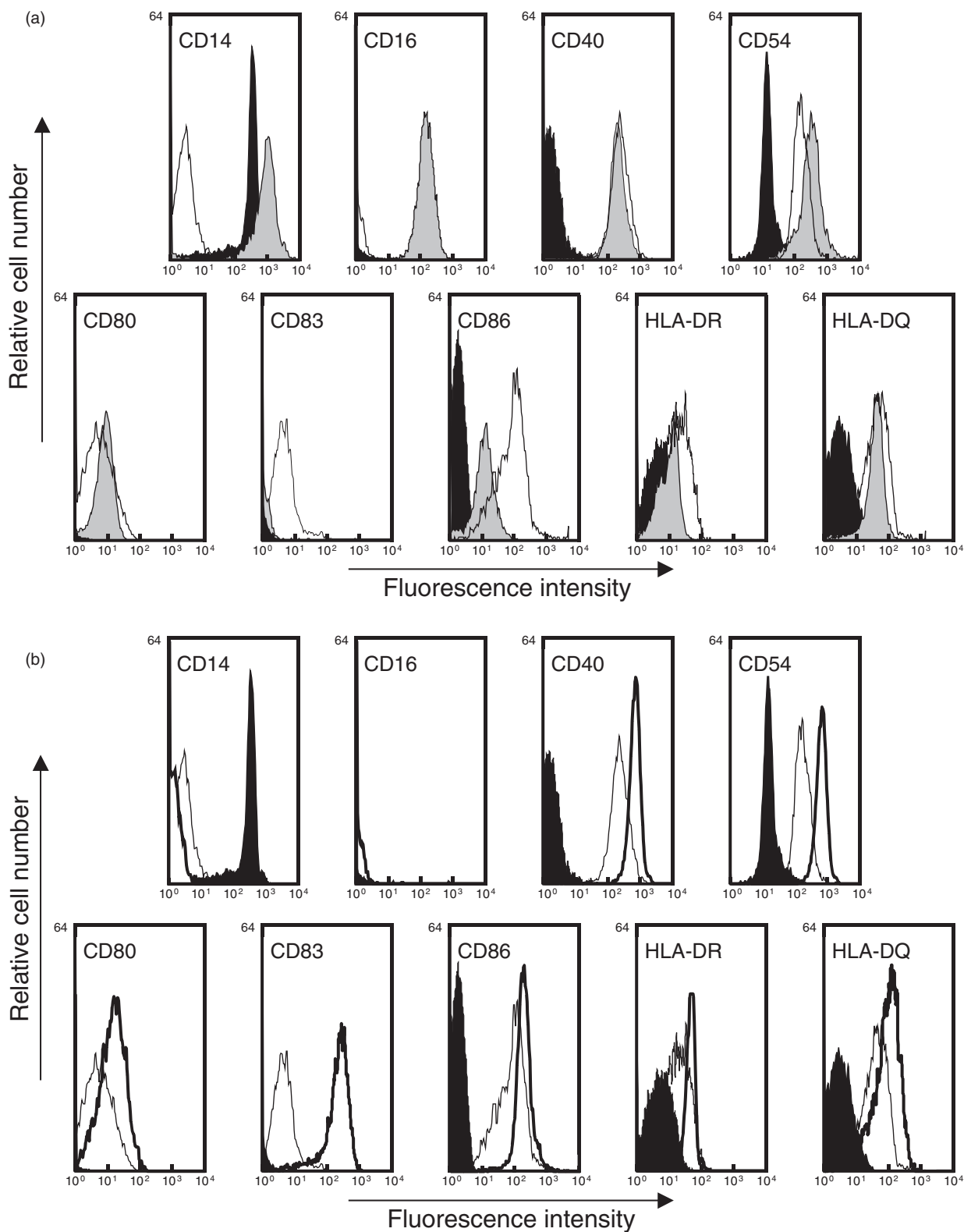


Fig. 1. Phenotypic characterization of the monocyte-derived cells generated in the absence of butyrate. Immature DC and MΦ were generated from monocytes cultured for 7 days in the presence of GM-CSF plus IL-4 or GM-CSF alone, respectively. Mature DC were derived from 5-day immature DC cultured for additional 2 days with TNF- α plus PGE₂. On day 7, cells were harvested and analysed using flow cytometry. (a) Immature DC (white profile) and MΦ (grey profile) were compared to freshly isolated monocytes (black profile) from the same individual. (b) Immature (white profile, thin line) and mature DC (white profile, bold line) were compared to monocytes (black profile). Data shown are representative of three separate experiments performed with three different donors.

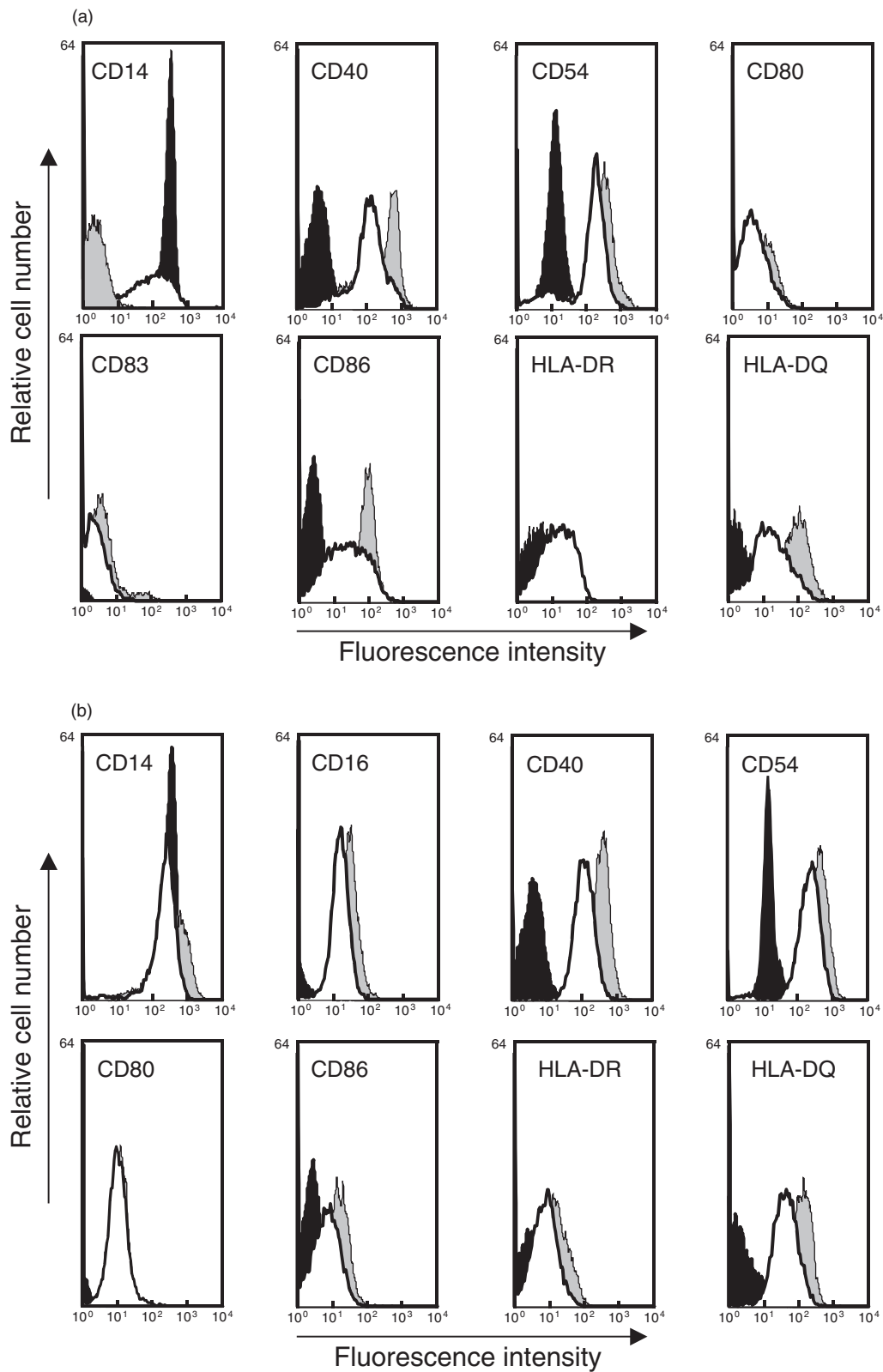


Fig. 2. Effect of butyrate on the phenotypic features of 7-day cultured DC and MΦ differentiation. Immature DC and MΦ were generated from monocytes cultured in the presence of GM-CSF plus IL-4 or GM-CSF alone, in the absence or in the presence of 0.5 mM butyrate. On day 7, cultured cells were harvested and analysed using flow cytometry. Immature DC (a) and MΦ (b) differentiated in the absence (grey profile) or in the presence (white profile) of 0.5 mM butyrate were compared to freshly isolated monocytes from the same individual (black profile). Data shown are representative of three separate experiments performed with three different donors.

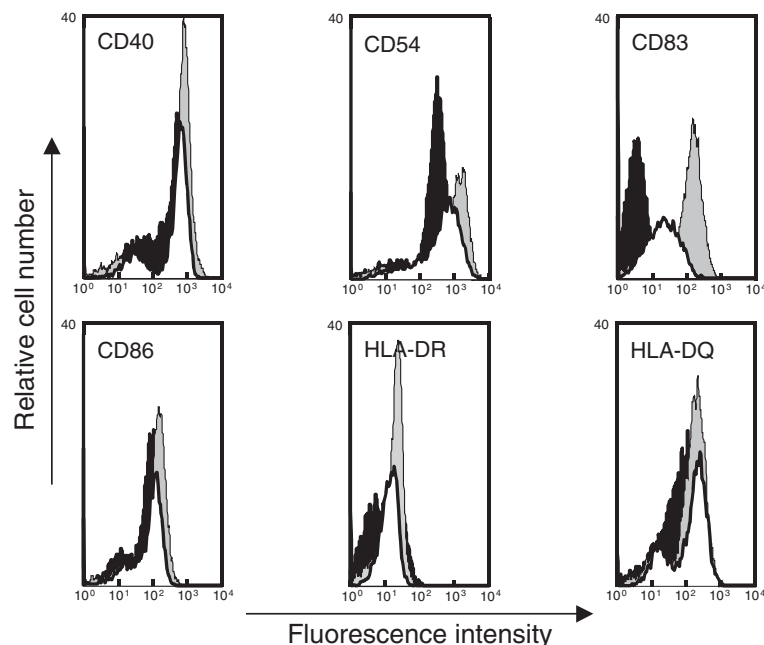


Fig. 3. Effect of butyrate on the phenotypic changes induced during DC maturation. Day 5 immature DC were cultured for 2 additional days with TNF- α plus PGE₂ in the absence (grey profile) or in the presence (white profile) of 0.5 mM butyrate. On day 7, cells were analysed by flow cytometry and compared to day 7 immature DC from the same individual (black profile). Data shown are representative of three separate experiments performed with three different donors.

microspheres, respectively. Butyrate also inhibited monocyte differentiation into M Φ , because 64% of treated cells engulfed at least 1 and 37% more than 3 microspheres, respectively (Fig. 5c).

Butyrate down-regulates T cell stimulatory activity of DC

The ability to prime T cells constitutes a unique and critical DC function that is related to the persistent expression of high levels of membrane MHC products and co-stimulatory molecules [28]. Therefore, as butyrate was found to down-modulate the expression of these antigens, we examined its effect on the ability of immature and mature DC to induce the MLR, a model for graft rejection in which DC are the most potent cells for priming naive T cells to alloantigens [27]. The study was performed using various APC/T cell ratios ranging from 1/10 to 1/1000 (Fig. 6). As expected, mature DC and to a lesser extent immature DC showed a strong capacity to activate T lymphocyte proliferation. In both cases, DC grown in the presence of butyrate exhibited reduced allostimulatory capability, as the allogeneic proliferation induced by treated cells was threefold and 1.5-fold less important than that induced by control immature and mature DC, respectively. This inhibitory effect of butyrate was more marked, for immature DC whose activity was nearly abrogated at the lower APC/T cell ratios ($P < 0.01$). These results indicate that the phenotypic changes induced by butyrate at key stages of DC differentiation correlate with the decreased allostimulatory properties of treated cells.

Butyrate synergistically acts with 1,25(OH)₂D₃ on DC phenotypic differentiation

As 1,25(OH)₂D₃ was shown to interfere with monocyte differentiation [29,30], we therefore studied the individual and combined

effect of butyrate and 1,25(OH)₂D₃ on the phenotypic differentiation of monocytes into DC. As shown in Fig. 7a, treatment with either butyrate or 1,25(OH)₂D₃ resulted in a preserved CD14 expression (mean fluorescence intensity: 13 ± 2 and 41 ± 9 , respectively) when compared to untreated cells (0.7 ± 0.1). Furthermore, combination of both treatments led to a synergistic effect on CD14 expression resulting in differentiated cells which exhibited a twofold increased CD14 membrane density when compared to fresh monocytes. In addition, Fig. 7b shows that combined treatment further reduced CD86 and HLA-DR expression, when compared to cells treated with each drug alone.

DISCUSSION

To our knowledge, this study represents the first to examine the effect of short-chain fatty acid butyrate on the differentiation and maturation process of DC and M Φ from monocytes. We demonstrate that butyrate partially inhibited the cytokine-driven phenotypic differentiation process of both cells, thus modulating their antigen capture efficiency. Moreover, butyrate altered the terminal maturation of DC and, consequently, profoundly decreased their ability to prime alloreactive naive T cells. Because DC have the unique property to induce primary immune response, and because M Φ are a major component of the innate and adaptive immune responses [31], suppression of DC and M Φ APC function by butyrate may represent a new, promising approach in immunotherapy.

Upon stimulation with GM-CSF and IL-4, monocytes differentiated into DC displaying phenotypic and functional characteristics. Compared to precursor monocytes, DC down-regulated

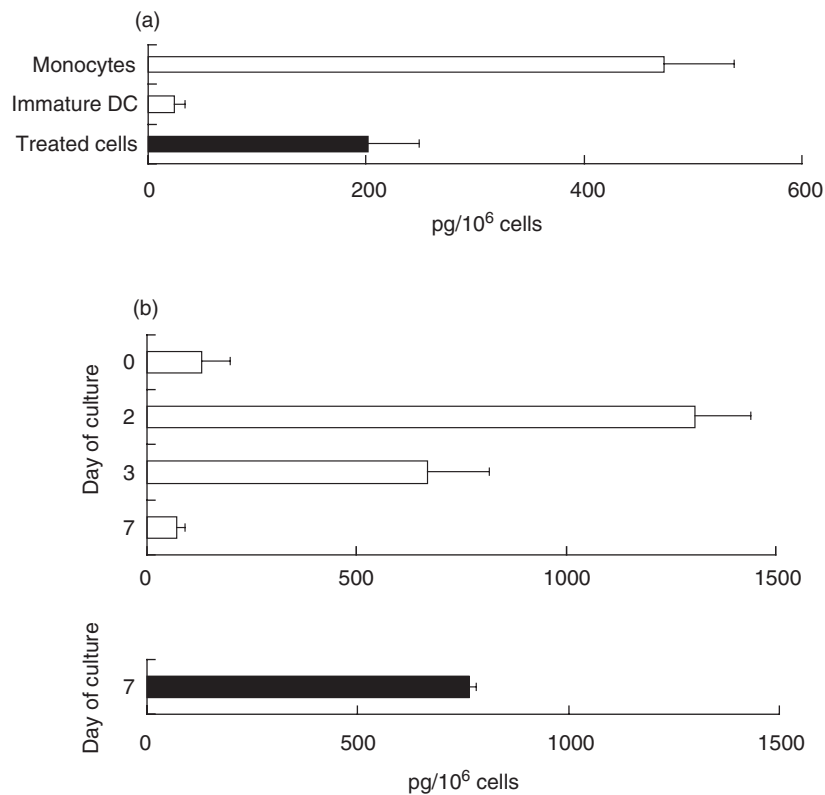


Fig. 4. Effect of butyrate on DC cytokine release. Immature DC were generated from monocytes in the absence or in the presence of 0.5 mM butyrate and assessed for their cytokine production. (a) IL-10 production by cells activated for 24 h with SAC strain. Results are expressed as mean pg/10⁶ cells \pm s.e.m. of six separate experiments performed with six different donors. (b) IL-12 production by cultured DC harvested at days 2, 3 or 7 and activated subsequently for 24 h with SAC strain plus IFN- γ . Results are expressed as mean pg/10⁶ cells \pm s.e.m. of six separate experiments performed with six different donors.

CD14, neoexpressed adhesion and co-activation molecules and up-regulated antigen-presentation molecules. In the presence of butyrate this phenotypic differentiation was inhibited partially, as we observed a persistence of CD14 and a decreased CD54, CD86 and HLA class II expression. This more immature differentiation stage was assessed further by the increased phagocytic capacity of the treated cells whose bead-engulfing capability was found intermediary between those of monocytes and untreated DC. Similarly, DC differentiated in the presence of butyrate exhibited an altered capacity to produce both IL-10 and IL-12 and developed only weak allostimulatory capacities.

Moreover, DC triggered to maturation through TNF- α plus PGE₂ in the presence of butyrate were unable (1) to express CD83, a marker highly restricted to mature DC, (2) to acquire highly level of co-stimulatory and antigen-presentation molecules such as CD40, CD86 and HLA class II and (3) to optimally stimulate allogeneic naive T cells. This effect of butyrate was independent of the type of maturation inducers used, because similar changes were observed with LPS or TNF- α plus IL-1 β .

Similarly, butyrate was found to alter the monocyte-derived M Φ differentiation, reducing the expression of restricted membrane antigens and the phagocytic capacity of treated cells. Overall, our data demonstrate clearly that butyrate modulates the differentiation potential of monocytes and leads to the generation of DC and M Φ with altered function. Whether butyrate simply blocks the dual monocyte differentiation or rather redirects this

process towards distinct functional programmes is still a matter of speculation.

Previous studies have shown that pretreatment of fresh monocytes with butyrate down-regulates their ability to stimulate T cell responses [14,32]. Altered APC function was considered to result from a down-regulation of distinct adhesion and/or co-stimulatory receptors on monocytes. However, these effects were observed only at high T cell/APC ratios and, more importantly, at high butyrate concentrations (1–2 mM), and such concentrations were shown by us and others [14] to result in a high level of monocyte death and apoptosis. At lower concentrations, butyrate did not significantly alter cell viability but virtually no APC suppression effect was observed in the fresh monocyte model [14]. Our results, obtained with a cultured cell model at a non-toxic butyrate concentration (0.5 mM), demonstrate a novel immune suppression property of butyrate that results in monocyte inability to differentiate into potent professional APC.

The active metabolite of the immunomodulatory hormone vitamin D₃, 1,25(OH)₂D₃, has also been shown to inhibit cytokine-driven differentiation, maturation and function of DC [30,33]. In addition, it has been demonstrated that butyrate in combination with 1,25(OH)₂D₃ acts synergistically on the differentiation of several neoplastic cell lines [34–36]. We therefore decided to study the effects of these treatments, alone or in combination, on the phenotypic changes induced by monocyte differentiation into DC in our physiologically more relevant model.

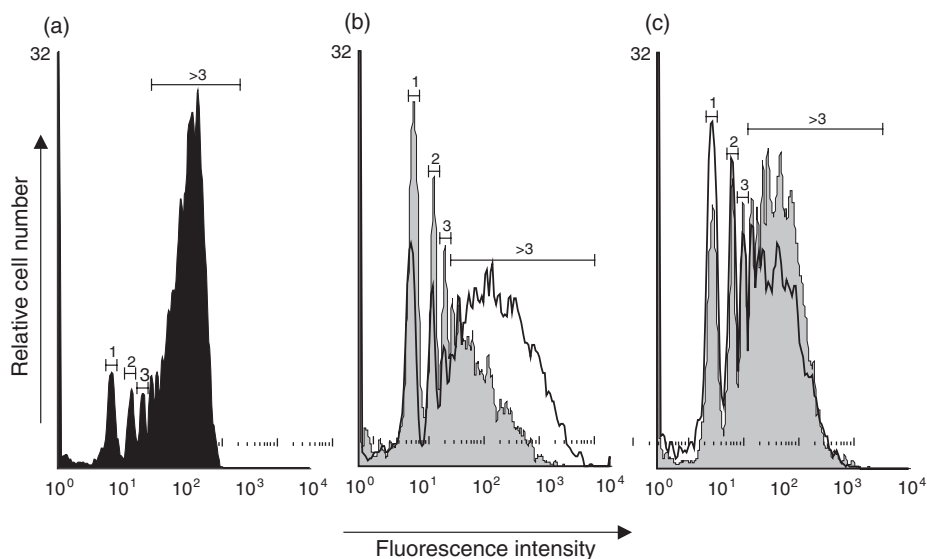


Fig. 5. Effect of butyrate on the phagocytic activity of immature DC and M Φ . Immature DC and M Φ were generated from monocytes cultured with GM-CSF plus IL-4 or GM-CSF alone, in the absence or in the presence of 0.5 mM butyrate. On day 7, the antigen capture capacity of immature DC (b) and M Φ (c) was evaluated using flow cytometry to assess single cell fluorescent microsphere engulfment and compared to data obtained with freshly isolated monocytes (a). The histograms of FITC-latex bead capture show a series of fluorescence peaks corresponding to clusters of cells that phagocytized 1, 2, 3 ... beads. Numbers above peaks indicate the number of ingested particles per cell. (a) Fresh monocytes (black profile) showed a high capture capacity assessed by a majority of cells that phagocytized more than three beads. (b) This phagocytosis activity is down-regulated upon differentiation into immature DC (grey profile) as demonstrated by the decreased number of cells that engulfed more than three beads. The presence of butyrate inhibits this phenomenon and leads to DC (white profile) with intermediate capture capacity. Conversely, phagocytosis activity of monocytes is up-regulated upon differentiation into M Φ (grey profile) as shown by the shift of the peak of cells engulfing a high number of beads. Butyrate partially inhibited this phenomenon (white profile). Data shown are representative of three separate experiments performed with three different donors.

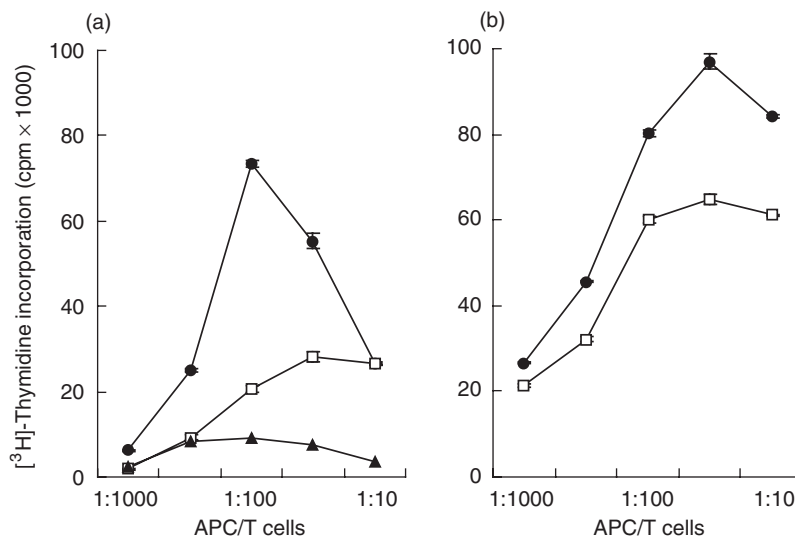


Fig. 6. Effect of butyrate on the T-cell allostimulatory capacity of DC. Immature DC were generated from monocytes cultured with GM-CSF plus IL-4 in the absence or in the presence of 0.5 mM butyrate. In a second set of experiments, 5-day immature DC were further matured for 48 h with TNF- α plus PGE₂. On day 7, cells were harvested and used as stimulators of allogeneic T cell proliferation. Thymidine incorporation was measured on day 5 by an 18-h pulse with [³H]-thymidine. (a) Immature DC were compared to freshly isolated monocytes from the same individual. (b) Mature DC. Data from quadruplicate assays are representative of three separate experiments performed with three different donors. (a) ●, Immature DC; □, treated cells; ▲, monocytes. (b) ●, Mature DC; □, treated cells.

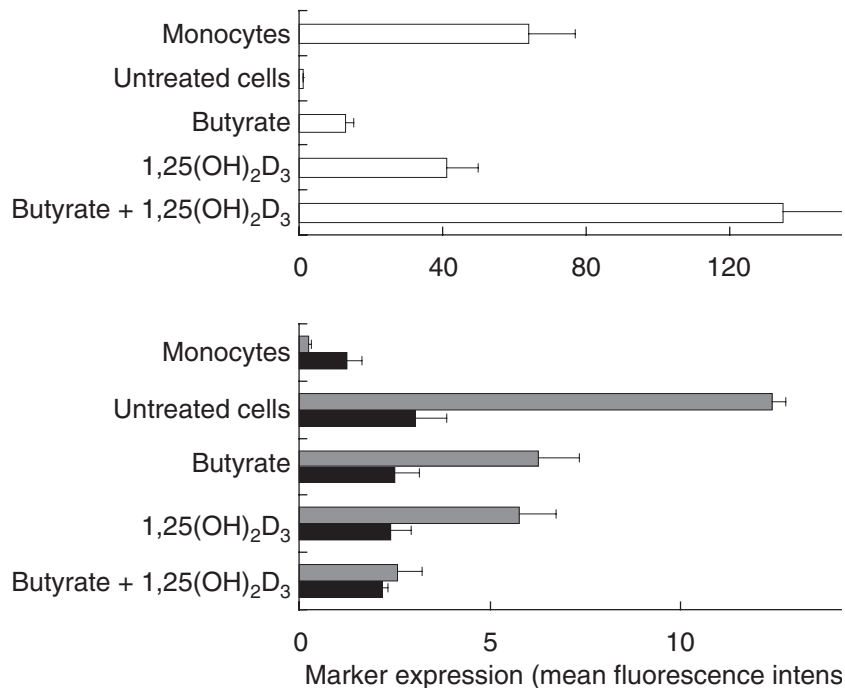


Fig. 7. Effect of treatment with butyrate and 1,25(OH)₂D₃ on the phenotypic differentiation of monocytes into DC. DC were generated from monocytes cultured in the presence of 0.5 mM butyrate, 10⁻⁷ M 1,25(OH)₂D₃ or a combination of both drugs, respectively. On day 7, cells were analysed for CD14, CD86 and HLA-DR expression using flow cytometry and compared to untreated cells and fresh monocytes from the same individual. Data shown are the mean ± s.e.m. of three separate experiments performed with three different donors. □, CD14; ■, CD86; ▀, HLA-DR.

Both butyrate and 1,25(OH)₂D₃ show an inhibitory effect on monocyte differentiation into DC, as assessed by the evolution of the phenotypic profile observed (Fig. 7). Moreover, when combined these treatments resulted in an additive effect on the inhibition of CD86 and HLA-DR expression, and a synergistic effect on CD14 expression which membrane density even exceeded that observed on fresh monocytes. Gaschott *et al.* [37] have suggested that the synergistic effect of butyrate and 1,25(OH)₂D₃, observed on tumoral cell lines could be due to an up-regulation of vitamin D receptors by butyrate. However, despite a similar synergistic effect on DC differentiation when both treatments were combined, 1,25(OH)₂D₃ and butyrate have been shown to alter monocyte differentiation in a different way, when used alone. 1,25(OH)₂D₃ has been shown to skew monocyte differentiation away from antigen-presenting DC development and towards the direction of mature phagocytosing MΦ [29], when butyrate inhibits monocyte differentiation into DC without redirecting towards MΦ (Fig. 2). Moreover, 1,25(OH)₂D₃ has been shown to promote the differentiation of monocytes towards MΦ [30], while in our study butyrate prevents both DC and MΦ differentiation (Fig. 2). Therefore, our results suggest that, at least in part, another mechanism might explain the effect of butyrate on the dual differentiation of monocytes.

Among other mechanisms by which butyrate could modulate differentiation and maturation, one can speculate a possible role for the NF-κB/Rel transcription factor family. NF-κB, identified initially as a constitutive immunoglobulin κ light-chain enhancer-binding protein present in the nuclei of B lymphocytes [38], has been found to play a central role in regulating the expression

of various cytokines and cell adhesion molecules involved in immune and inflammatory responses [39]. Recently, gradual differences in the DNA binding activities of different NF-κB homo- and heterodimers have been shown to correlate with differentiation stages of monocytes, MΦ and DC [40]. In the same way, butyrate has been shown to inhibit the activation of NF-κB on cultured cells [41]. This inhibitory effect could result from direct alteration of the expression of one or more genes that function subsequently to regulate NF-κB activity, but also from inhibition of NF-κB activation by TNF-α [41]. Therefore, inhibition of NF-κB activity by butyrate is likely to have a role in impaired DC and MΦ differentiation and DC maturation.

Butyrate or related compounds have recently been suggested to have potential clinical relevance for the treatment of autoimmune diseases as well as for the inhibition of alloresponses [2,3,42]. Butyrate enemas or a high-fibre diet that increase colonic butyrate concentrations have been shown to be effective treatments for mucosal inflammation in both humans and animal models of colitis [43–47]. In addition, supplementation of drinking water with butyrate resulted in a modest, but significant prolongation of skin allograft survival in mice [1]. The therapeutic potential of butyrate, which is limited by its short half-life *in vivo* [48], has been improved by the synthesis of prodrugs of n-butyric acid extending the residence time of potentially releasable n-butyric acid *in vivo* [3,49–53]. These compounds have been shown to retain the majority of the biological properties *in vitro* as well as *in vivo* [32,54–57]. Evidence for an anti-inflammatory effect of these drugs on human monocytes [42], as well as prolongation of heart allograft survival in animals [2], have been obtained. Our

results, demonstrating impairment of DC and M Φ differentiation by butyrate, represent important clues to understand the mechanism of action of butyrate on both inflammatory and alloresponses, and support further the interest for butyrate and its derivatives as new immunotherapeutic reagents.

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