CCR5 in multiple sclerosis : expression, regulation and modulation by statins

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Statins & monocyte differentiation

Simvastatin inhibits differentiation of monocytes into immature DC and not DC maturation

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In preparation

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Abstract

Recently, statins have become subject of intense investigation because of their immunomodulatory capacities. However, the mechanisms by which statins act on the immune system are still poorly understood. Previously, it has been demonstrated that statins may affect antigen presentation functions of dendritic cells (DC), which play an essential role in the initiation of antigen-specific immune responses. However, it is not clear whether the effect of statins on antigen presentation functions of DC is due to interference of statins in the processes required for DC differentiation or for DC maturation. In this study, we have therefore examined the effect of simvastatin on the differentiation of blood-borne monocytes into DC and the maturation of these DC by evaluating cell surface expression of several well-known DC markers. The results of our studies reveal that simvastatin affects monocyte-DC differentiation and not DC maturation. Furthermore, we show that exposure to simvastatin during monocyte-DC differentiation affects the expression of costimulatory and MHC-II molecules on DC, which may have an impact on the initiation of antigen-specific immune responses.

Introduction

Statins are potent inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the key enzyme in cholesterol biosynthesis [1]. Because of their ability to inhibit the synthesis of cholesterol, statins are extensively used in medical practice as therapy for hypercholesterolemia and have been shown to lower cardiovascular-related morbidity and mortality [2,3]. In addition to the effect of statins on atherosclerosis, there is growing evidence suggesting that statins have potential immunomodulatory capacities. For example, statins are able to inhibit the production of pro-inflammatory cytokines [4-7], lower the expression of major histocompatibility complex (MHC) class I and II molecules and other immunomodulatory molecules on various cell types [6-9] and affect lymphocyte, dendritic cell and microglial cell functions *in vitro* [6,7,10,11].

Dendritic cells (DC) are the key players in the initiation of a primary immune response, due to their ability to present antigen to, and subsequently activate T-cells [12,13]. *In vivo*, DC are distributed ubiquitously in peripheral tissues in an immature state, which is characterized by low membrane expression of MHC-II molecules and lack of expression of the costimulatory molecules CD40, CD80 and CD86. Immature DC display a very efficient antigen uptake capacity, after which antigens are processed and presented at the cell surface as peptides in the context of MHC-II molecules. This maturation process is accompanied by upregulation of MHC-II and costimulatory molecules that are needed for adequate antigen-specific T-cell activation [12,13]. *In vitro*, DC can be generated from blood-borne monocytes [14]. During 3 to 5 days of culture in the presence of GM-CSF and IL-4, monocytes differentiate into immature DC. Because of their immature phenotype, characterized by low levels (or lack) of expression of MHC-II and costimulatory molecules, these *in vitro* generated immature DC are capable of endocytosis, but unable to activate T cells. In this way, they resemble immature DC that reside in peripheral tissue under normal conditions. *In vitro*, these generated immature DC can be matured by various stimuli such as microbial compounds (e.g. lipopolysaccharide (LPS)), inflammatory cytokines (e.g. TNF-α) or T-cell signals (e.g. CD40L) and become potent activators of T-cells due to upregulation of MHC and costimulatory molecule expression and concomitant reduction of endocytosis activity [14].

Several studies have investigated the effect of statins on DC function. These studies show that statins affect the ability of DC to present antigens to, and activate T-cells [6,15]. This reduced antigen-presenting capacity and resulting lack of T-cell activation could result from the reported inhibitory effect of statins on monocyte-derived or bone-marrow-derived DC maturation [15-17]. However, these reports do not discriminate whether statins affect differentiation of monocytes into immature DC, or whether statins affect maturation of immature DC.

Our current study reveals that the statin simvastatin affects monocyte-DC differentiation and not maturation of these immature monocyte-derived DC into

mature DC. Furthermore, we show that exposure to simvastatin during monocyte-DC differentiation has an effect on the capacity of DC to express costimulatory and MHC-II molecules, which may have an impact on the initiation of antigen-specific immune responses.

Materials & Methods

Simvastatin activation

Simvastatin was obtained from Calbiochem (Darmstadt, Germany). Prior to use, simvastatin was converted to its active form. Briefly, 12,5 mg of simvastatin was dissolved in 250 μl of ethanol and 203 μl of 1M NaOH was added. After this, pH was adjusted to 7,2 and the volume was corrected to 3 ml, resulting in a 10 mM stock. Concentrations of simvastatin applied and times of exposure are indicated in the figure legends.

Generation of dendritic cells (DC) from monocytes

Peripheral blood mononuclear cells (PBMC) were isolated from blood of normal healthy donors using a Ficoll gradient (Pharmacy Leiden University Medical Center, Leiden, The Netherlands). To obtain monocytes, cells were left to stand in a tissue culture flask in RPMI-1640 medium (Life Technologies, Breda, The Netherlands) supplemented with 10% (v/v) heat-inactivated FCS, 100 IU/ml streptomycin, 100 IU/ml penicillin and 2mM L-glutamate for at least 1 hour to let monocytes adhere to the culture flask. After this, non-adherent cells were washed away and monocytes were stimulated with a combination of 1000 U/ml recombinant human granulocyte macrophage stimulating factor (GM-CSF) and 1000 U/ml IL-4 (both BioSource, Nivelles, Belgium), for 5 days to obtain immature DC. For differentiation experiments, cells were treated with 10 μM simvastatin during the differentiation period.

DC maturation

After differentiation of monocytes into DC, immature DC were matured by stimulating the cells with 100 ng/ml LPS from *Escherichia coli* (Sigma-Aldrich, St. Louis, MO) or 500 u/ml TNF-α (R&D Sytems, Minneapolis, MN) for 2 days. For maturation experiments, cells were incubated with 10 μM simvastatin either during the maturation period (simultaneously with LPS/TNF-α stimulation) or during differentiation of monocytes to DC, after which simvastatin-treated DC were matured with LPS or TNF-α.

Flow cytometric analysis

Cell surface expression of DC (maturation) markers was evaluated by flow cytometry. Cells were stained with R-phycoerythrin (PE)-conjugated mouse monoclonal antibodies

against CD1a, CD11b, CD11c, CD14, CD40, CD54, CD64, CD80, CD83, CD86 and HLA-DR (Becton Dickinson, San Jose, CA). As controls, cells were stained with PE-conjugated IgG1, IgG2a or IgG2b. Wash steps were performed with PBS containing 1% FCS. After staining and washing, cells were fixed with 1% paraformaldehyde in PBS. Fluorescence-activated cell sorting (FACS) analysis was performed on a FACSCalibur flow cytometer (Becton Dickinson) using Cell Quest programming.

Results

Simvastatin affects differentiation of monocytes into immature DC and not DC maturation

To investigate the effect of simvastatin on monocyte-DC differentiation, monocytes derived from PBMC were differentiated *in vitro* into immature DC in the presence of GM-CSF and IL-4 for 5 days. Differentiation of monocytes into immature DC was confirmed by loss of CD14 and CD64 expression (not shown) and induction of expression of the DC marker CD1a (Figure 1) as determined by FACS analysis. In addition to high expression of CD1a, these immature monocyte-derived DC expressed high levels of the integrins CD11b and CD11c and the adhesion molecule CD54

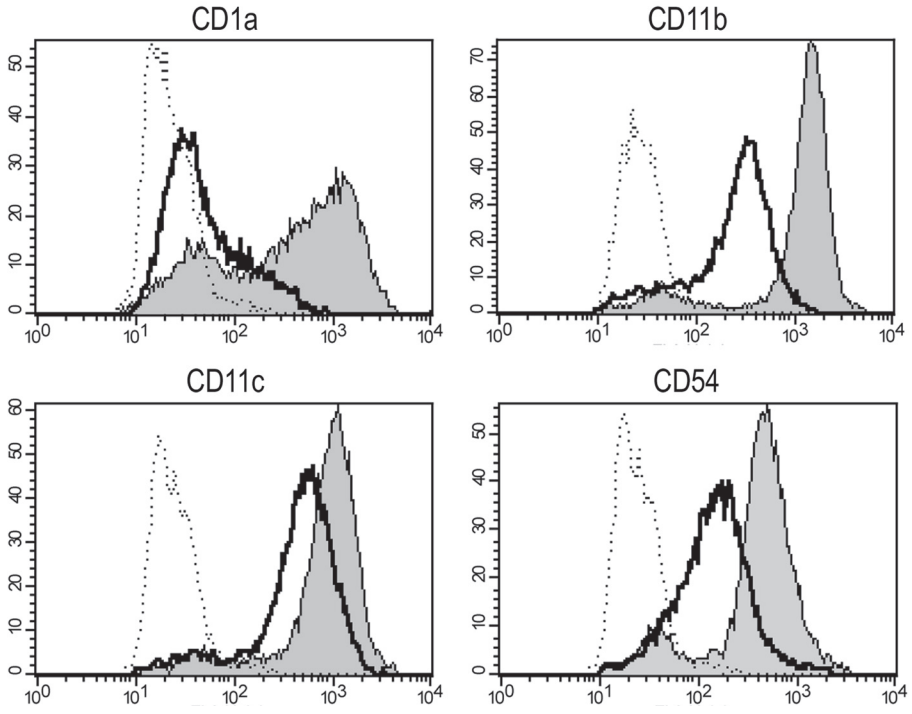

Figure 1. Simvastatin inhibits differentiation of monocytes into immature DC. FACS analysis of cell surface expression of CD1a, CD11b, CD11c and CD54 on monocyte-derived immature DC. Histograms depict cell surface expression of the investigated markers on normally differentiated cells (filled histograms) and monocytes differentiated in the presence of simvastatin (10 μM; thick line). Dotted lines represent isotype control staining. Shown are representatives of 3 independent experiments.

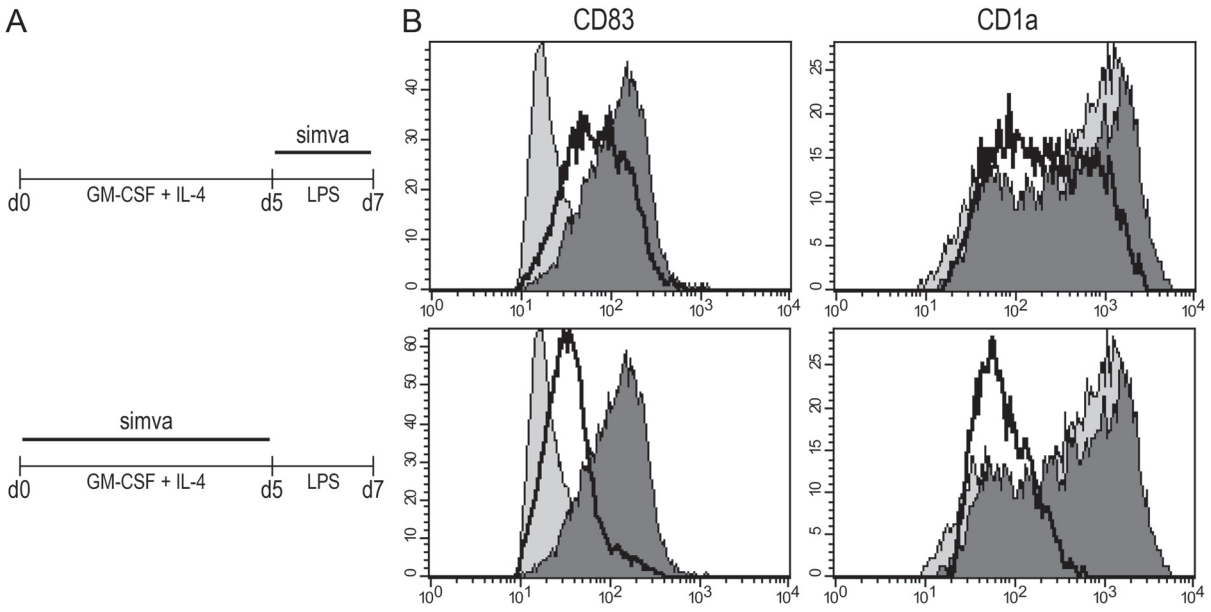

Figure 2. Simvastatin partially inhibits DC maturation. FACS analysis of cell surface expression of CD83 and CD1a on immature monocyte-derived DC stimulated with LPS (100 ng/ml, 48 hours). **A** Schemes depicting the different treatment regimes applied. The upper panel depicts simvastatin (10 μM) treatment during the maturation period (days 5 to 7). The lower panel illustrates simvastatin treatment during the differentiation phase (days 0 to 5), followed by normal maturation in the absence of simvastatin. **B** Histograms depict cell surface expression of CD83 (left panels) and CD1a (right panels) on immature DC (light grey histograms), mature DC (dark grey histograms) and simvastatin treated mature DC (thick line). Upper panels compare cell surface expression on normally matured DC with DC treated with simvastatin during maturation. Lower panels compare cell surface expression of CD83 and CD1a on normally matured DC with monocytes treated with simvastatin during differentiation. Shown are representatives of 3 independent experiments.

(ICAM-1) (Figure 1). In contrast, these immature DC lacked expression of CD83 and costimulatory molecules (not shown).

To assess the effect of simvastatin on monocyte-DC differentiation, we compared the expression of the markers mentioned above on normally differentiated monocytes to that on monocytes treated with simvastatin during differentiation. Exposure to simvastatin during differentiation blocked the induction of CD1a expression on immature DC (Figure 1). In addition, we observed an inhibitory effect of simvastatin on the expression of CD11b, CD11c and CD54 on immature DC (Figure 1). However, the extent of this downregulatory effect of simvastatin varied between the different molecules, from a lesser effect on CD11c expression to a marked downregulation of CD11b and CD54 expression.

Next, we investigated the effect of simvastatin on DC maturation. For this, we monitored the expression of the DC maturation marker CD83. Immature DC did not express CD83. However, expression of CD83 was induced when immature monocyte-derived DC were exposed for 2 days to the strong microbial inducer of maturation LPS (Figure 2), or the inflammatory cytokine TNF-α (not shown). Simvastatin treatment during this maturation period only slightly interfered with the induction of CD83 expression on DC (Figure 2, upper panel). In contrast, when simvastatin was applied

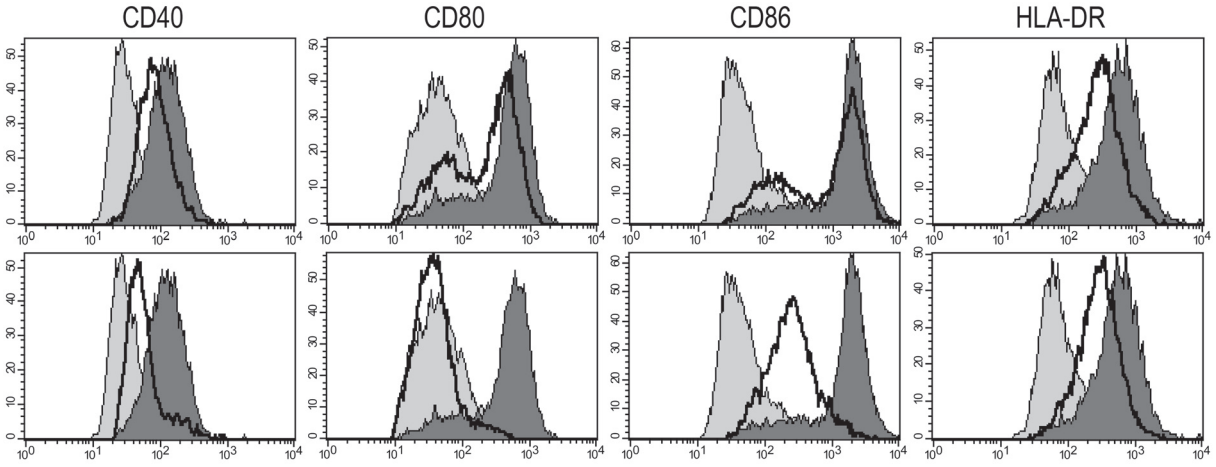

Figure 3. Simvastatin differentially affects costimulatory molecule and MHC-II expression on DC. FACS analysis of cell surface expression of costimulatory and MHC-II molecules on monocyte-derived DC stimulated with LPS (100 ng/ml, 48 hours). Histograms depict cell surface expression of CD40, CD80, CD86 and HLA-DR on immature monocyte-derived DC (light grey histograms), mature DC (dark grey histograms) and simvastatin treated mature DC (thick line). Schemes depicting the different treatment regimes are presented in Figure 2A. Upper panels compare cell surface expression on normally matured DC with DC treated with simvastatin (10 μM) during maturation. Lower panels compare cell surface expression of the various markers on normally matured DC with DC treated with simvastatin during differentiation, after which cells were matured normally. Shown are representatives of 3 independent experiments.

during the differentiation phase prior to maturation, immature DC failed to induce CD83 expression upon maturation (Figure 2, lower panel). Furthermore, simvastatin treatment during maturation did not or hardly interfere with the expression of CD1a, CD11b, CD11c and CD54, whereas simvastatin pretreatment during monocyte differentiation resulted in markedly decreased expression of these molecules (Figure 2 and not shown).

Together, our data show that simvastatin almost completely inhibits CD1a upregulation during differentiation and blocks maturation-induced CD83 expression when applied in the differentiation phase, whereas simvastatin treatment in the maturation phase has no clear effects. Therefore these finding reveal that simvastatin interferes mostly in the differentiation of monocytes into immature DC and not, or only partly, in DC maturation.

Simvastatin affects acquisition of antigen presentation functions of DC

We also investigated the effect of simvastatin on the maturation-induced acquisition of antigen presenting functions of DC by studying the expression of costimulatory molecules (CD40, CD80 and CD86) and of the MHC-II molecule HLA-DR. To investigate the effect of simvastatin on this process, we exposed monocyte-derived DC to simvastatin either during the maturation phase or during the differentiation phase (after which simvastatin-treated cells were matured normally) and monitored costimulatory molecule and MHC-II expression by FACS analysis.

Expression of CD40, CD80, CD86 and HLA-DR was low or absent in non-treated immature DC (Figure 3, light grey histograms) and was upregulated during maturation (Figure 3, dark grey histograms). Simvastatin treatment during the maturation phase affected the expression of the costimulatory molecules CD40, CD80 and CD86 only slightly, or only in a small population of cells (Figure 3, upper panels, thick lines). The same was observed for HLA-DR expression. In contrast, exposure to simvastatin during the differentiation phase inhibited CD40 and CD80 upregulation almost completely (Figure 3, lower panels). Remarkably, simvastatin treatment during differentiation, followed by normal maturation, resulted in intermediate expression of CD86 and HLA-DR (Figure 3, lower panels).

Taken together, these results reveal differential effects of simvastatin on the cell surface expression of costimulatory molecules and MHC-II during differentiation and maturation

Differential effects of simvastatin on HLA-DR and CD86 expression during differentiation

As mentioned above, the effect of simvastatin on CD86 and MHC-II expression contrasted with that on the expression characteristics of the investigated other (maturation) makers (Figure 3). Therefore, we also assessed expression of CD86 and HLA-DR during differentiation of monocytes into DC, and the effect of simvastatin on this expression.

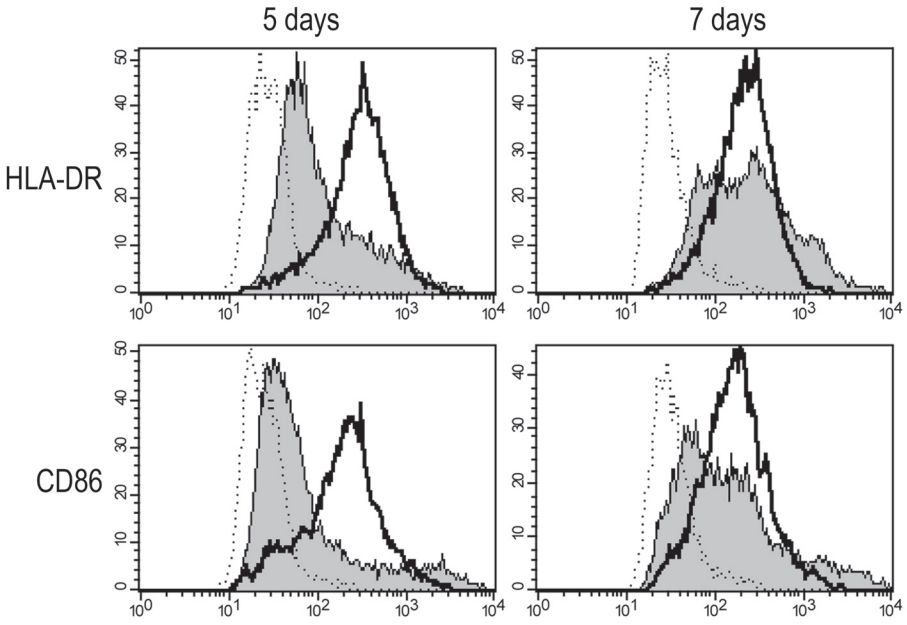

Figure 4. Simvastatin differentially affects expression of MHC-II and CD86 during differentiation. FACS analysis of HLA-DR and CD86 cell surface expression on monocyte-derived DC after 5 days (left panels) and 7 days (right panels) of culture in differentiation medium (GM-CSF/IL-4). Cell surface expression of HLA-DR and CD86 on normally differentiated DC (filled histograms) is compared with that of cells treated with simvastatin (10 μM) during differentiation (thick line). Dotted lines represent isotype control staining. Shown are representatives of 3 independent experiments.

Normally differentiated immature DC expressed low levels of HLA-DR and CD86 on the cell surface (Figure 4, left panels, filled histograms). In contrast to the other markers investigated, when monocytes were treated with simvastatin during differentiation (5 days), cell surface expression of HLA-DR and CD86 was enhanced compared to normally differentiated monocytes (Figure 4, left panels, thick lines).

When normally differentiated cells were kept on differentiation medium for 2 additional days, DC acquired a somewhat early maturation state, characterized by intermediate cell surface expression of HLA-DR and CD86 (Figure 4, right panels, filled histograms). Interestingly, simvastatin treatment during this prolonged (7 day) differentiation period did not enhance or reduce to a major extent this expression, resulting in a level of HLA-DR and CD86 expression that was comparable to that observed after 5 day-differentiation in the presence of simvastatin.

Taken together, these data suggest that early in differentiation, when membrane expression of HLA-DR and CD86 is low, simvastatin treatment enhances this expression, whereas late in differentiation (or early in maturation), when normally differentiated DC already express HLA-DR and CD86 on their cell surface, simvastatin does not affect this expression.

Discussion

In the current study, we have examined the effect of simvastatin on the differentiation of monocytes into DC and the maturation of these monocytes-derived DC. The present study is the first report of the effects of simvastatin on the generation of DC from blood-borne monocytes. We observed that simvastatin treatment renders monocytes unable to upregulate CD1a expression, which is a characteristic marker for differentiation of monocytes into DC. In addition, expression of other molecules, important for DC functioning, such as integrins and adhesion molecules, is reduced by simvastatin during differentiation. Therefore, it seems that simvastatin prevents monocytes from developing into functional immature DC.

In addition to this, we examined the effect of simvastatin on maturation of monocyte-derived DC. We found that simvastatin is unable to inhibit DC maturation, affecting expression of DC maturation markers only partly. This is in contrast to the effect of simvastatin treatment when it is applied before maturation, during monocyte differentiation into immature DC, completely blocking the upregulation of CD83 and the costimulatory molecules CD40 and CD80. Taken together, our results show that simvastatin does inhibit monocyte-DC differentiation in vitro, rendering the resulting cells unable to further mature, but does not, or only partly, inhibit maturation of immature DC.

Recently, two groups have reported effects of statins on DC maturation. Seemingly in contrast to our findings, Yilmaz *et al*. have reported that statins do inhibit monocyte-derived DC maturation, reducing cell surface expression of maturation markers to a

level similar to expression on immature or early mature DC [16]. However, the effect they investigated was that of statin pretreatment prior to maturation and not during the maturation phase itself. Therefore, their findings corroborate ours that simvastatin treatment of DC before the maturation phase renders cells unresponsive to maturation signals. Additionally, we show that statins, when given simultaneously with maturation signals, do not have a very potent effect on maturation, which is in line with the findings of Sun *et al.*, who describe a partial inhibition of murine bone-marrow-derived DC maturation by statins [15].

With regard to the effect of simvastatin on CD80 and CD86 upregulation during DC maturation, we found that simvastatin affects the upregulation of these molecules only on a subpopulation of maturing DC. A similar effect of simvastatin on CD86 expression on DC has been previously noted [16], revealing that the effect of simvastatin on DC is not homogenous. This suggests the existence of several populations of DC, with differential vulnerability to statin treatment. From the current experiments it is not clear whether the cells that are susceptible to simvastatin treatment consist of one population losing both CD80 and CD86 expression, or that cells randomly lose CD80 or CD86 expression. When compared to the effect of simvastatin on CD80 and CD86 expression, upregulation of CD40 and CD83 during maturation is less affected by simvastatin. Moreover, the effect of simvastatin on the expression of these molecules concerns the total population of maturing DC and is not confined to a subpopulation of cells. Therefore, assuming that the cells losing CD80 and CD86 expression represent a single population, it seems that the process of CD80/CD86 upregulation in these cells is more susceptible to statin treatment than the upregulation of CD40 and CD83 expression.

Another very interesting finding is that simvastatin treatment during differentiation of monocytes into DC results in a clear increase of HLA-DR and CD86 cell surface expression. A possible explanation for this observation is that simvastatin treatment initiates a kind of maturation process that leads to cell surface expression of antigen presentation molecules in the absence of maturation signals. This is corroborated by the finding that after 5 days of differentiation simvastatin-treated DC express HLA-DR and CD86 while normally differentiated cells do not (or at low levels), but after a prolonged differentiation period this expression is not further enhanced. The expression of HLA-DR and CD86 on simvastatin-treated DC is at that time point comparable to normally differentiated DC that develop a somewhat mature state during this extended differentiation period.

However, the hypothesis that simvastatin initiates DC maturation only holds with regard to HLA-DR and CD86 expression, because the expression of other maturation markers is not, or only minimally, enhanced by simvastatin. Several possible mechanisms can explain these findings. Firstly, it could be that this is because the kinetics of membrane expression of these molecules is much slower than that of HLA-DR and CD86. However, it is more likely that the effect of simvastatin on HLA-DR

and CD86 expression is not the result of an effect on (the rate of) maturation, but that simvastatin merely affects the localization of these molecules. Indeed, it is known that immature DC synthesize high numbers of MHC-II molecules, but retain these molecules in intracellular compartments [12,13,18]. During maturation, the localization of these molecules is dramatically changed to the cell membrane. Therefore, a second explanation of the effect of simvastatin on HLA-DR expression is that MHC-II molecules present in immature DC are retained in these endosomal compartments by a mechanism dependent on one or more products of the mevalonate pathway. By disturbing this pathway, simvastatin would inhibit the retention of MHC-II in endosomal compartments, resulting in enhanced transport of these molecules to the cell surface.

Thirdly, there is also evidence that immature DC, like mature DC, do express MHC-II on their cell surface [18]. In immature DC these molecules are quickly endocytosed "back" into the cell, resulting in a short half-life and effectively low numbers of MHC-II molecules on the cell surface, whereas on mature DC MHC-II molecules remain on the DC surface for extended periods, resulting in high membrane expression and enabling DC to activate T-cells also after a longer period of time and provide antigenic memory. It could be that the process of MHC-II internalization in immature DC is dependent on cholesterol or another product of the mevalonate pathway. If this is the case, simvastatin would inhibit the internalization of MHC-II molecules by immature DC, resulting in a longer half-life and accumulation of molecules on the cell surface. Whether CD86 surface expression is regulated by the same mechanism, is unclear. However, our data suggest that CD86 surface expression in DC parallels MHC-II expression.

In earlier studies, we have shown that simvastatin affects MHC-II membrane expression by disturbing the integrity of lipid rafts, microdomains which transport MHC-II molecules to the cell surface [8]. The fact that we now find that simvastatin enhances MHC-II expression seems contradictory to these earlier findings. However, Kropshofer *et al.* have shown that on professional APC, peptide-loaded MHC-II molecules are enriched in tetraspan microdomains [19]. These microdomains differ from lipid rafts in their composition and the fact that they enrich only MHC-II molecules that carry a selected set of peptides. The peptide-MHC-II complexes that are incorporated into tetraspan microdomains are not only localized on the cell surface, but also in internal compartments, where loading of antigen occurs. Indeed, it has been shown that in immature DC, HLA-DR molecules are located in internal tetraspan compartments [19]. Interestingly, CD86 molecules are localized in these same domains. Upon maturation, cell surface expression of these microdomains, and consequently HLA-DR and CD86 expression, is upregulated [19]. Possibly, simvastatin treatment of immature DC could disturb the mechanism that retains MHC-II/CD86 containing tetraspan compartments inside the cell, enabling these microdomains to localize to the cell membrane.

In conclusion, we demonstrate here that simvastatin inihibits monocyte-DC

differentiation and not maturation of immature DC. In addition, we show that simvastatin interferes with the acquisition of costimulatory and MHC-II molecule expression during these processes, which may affect T-cell stimulatory capacities of DC.

Acknowledgements

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