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

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Statins & MHC-II / immuno-regulatory molecules

Statins affect cell surface expression of major histocompatibility complex class II molecules by disrupting cholesterol-containing microdomains

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Abstract

Statins, the main therapy for hypercholesterolemia, are currently considered as possible immunomodulatory agents. Statins inhibit the production of pro-inflammatory cytokines and reduce the expression of several immunoregulatory molecules, including major histocompatibility complex class II (MHC-II) molecules. In this study, we investigated the mechanism by which simvastatin reduces the membrane expression of MHC-II molecules on several human cell types. We show that the reduction of MHC-II membrane expression by simvastatin correlates with disruption of cholesterol-containing microdomains, which transport and concentrate MHC-II molecules to the cell surface. In addition, we show also that statins reduce cell surface expression of other immunoregulatory molecules, which include MHC-I, CD3, CD4, CD8, CD28, CD40, CD80, CD86 and CD54. Our observations indicate that the down-regulation of MHC-II at the cell surface contributes to the immunomodulatory properties of statins and is achieved through disruption of cholesterol-containing microdomains, which are involved in their intracellular transport.

Introduction

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, also known as statins, are potent inhibitors of the endogenous mevalonate pathway and subsequent cholesterol biosynthesis [1]. Due to their ability to inhibit the synthesis of cholesterol, statins are widely used in medical practice and are the principal therapy for hypercholesterolemia. Indeed, statins have been shown to induce a regression in vascular atherosclerosis and a reduction in cardiovascular-related morbidity and mortality in patients with and without coronary artery disease [2-5]. In addition to the effect of statins on atherosclerosis, various findings suggest that statins also exert anti-inflammatory properties by inhibiting the production of several pro-inflammatory cytokines [6-10]. Recent studies have also shown that statins are able to inhibit the IFN-γ-mediated induction of major histocompatibility complex class II (MHC-II) molecules on various cell types [11-13], and the constitutive MHC-II expression on B lymphocytes [13]. However, the exact mechanism of the anti-inflammatory activity of statins remains to be defined.

Interaction between immune cells and signaling via T-cell receptors (TCRs) and MHC-II molecules occurs in specific microdomains on the plasma membrane. These membrane microdomains, which are highly enriched in cholesterol and glycosphingolipids, are known as detergent-insoluble glycolipid-enriched complexes (DIGs), glycosphingolipid-enriched membrane microdomains (GEMs) or lipid rafts [14-16]. Because of their particular biochemical en biophysical nature, lipid rafts are resistant to solubilization by non-ionic detergents and have a low buoyancy, properties that allow their separation by density-gradient centrifugation. An important role of lipid rafts is the recruitment and concentration of cell signaling molecules to the plasma membrane [15], a mechanism essential for T and B cell signaling [17-19]. Moreover, recent studies have shown that surface MHC-II molecules are also present in lipid rafts and that this association serves to increase the local concentration of MHC-II molecules in distinct cholesterol-containing plasma membrane microdomains in order to facilitate antigen presentation [20-24]. Therefore, inhibiting cholesterol biosynthesis by statins could affect the integrity of these microdomains [25].

In this study, we show that the statin simvastatin does indeed inhibit IFN-γ-induced MHC-II membrane expression in monocytic cell lines and several tumor cell lines, as well as MHC-II membrane expression in primary B lymphocytes and in primary activated T lymphocytes. Subsequently, we show that the inhibition of MHC-II expression by simvastatin results from the disruption of cholesterol-containing microdomains, which transport MHC-II molecules to and sustain their expression on the cell membrane. In addition, we show that simvastatin also affects cell surface expression of MHC-I molecules. Notably, besides MHC-I and MHC-II molecules, the expression of several other molecules that play important roles in immune activation is affected by simvastatin in human peripheral blood mononuclear cells. Altogether,

we provide evidence for a mechanism of immunomodulation by simvastatin, in which simvastatin disturbs the formation of cholesterol-containing microdomains, leading to the reduced expression of MHC-II and MHC-I molecules, which play an important role in the initiation and effector phases of antigen-specific immune responses.

Materials & Methods

Cell culture

The cell lines THP-1, U937, U251-MG, U373-MG, HeLa and Raji (all American Type Culture Collection, Manassas, Virginia) were cultured in Iscove's modified Dulbecco's medium (IMDM; BioWhittaker Europe, Verviers, Belgium) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS; Greiner, Alphen a/d Rijn, The Netherlands), 100 IU/ml streptomycin and 100 IU/ml penicillin. HH cells (ATCC) were cultured in RPMI-1640 (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. Where indicated, cells were stimulated with 500 U/ml IFN-γ (Boehringer-Ingelheim, Alkmaar, The Netherlands) with or without simvastatin (1-10 μM) (Calbiochem, Darmstadt, Germany) and 100 μM L-mevalonate (Sigma-Aldrich, Steinheim, Germany). Prior to use, simvastatin and L-mevalonate were converted to their active forms as described earlier [26,27].

PBMC isolation and stimulation

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). After isolation, cells were cultured in RPMI-1640 and stimulated with 1 μg/ml phytohaemagglutinin (PHA; Murex Biotech, Dartford, United Kingdom) for 3 days.

Flow cytometric analysis

Cells were stimulated with 500 U/ml IFN-γ with or without 10 μM simvastatin, or with 10 μM simvastatin and 100 μM L-mevalonate for 48 hours and stained with a R-phycoerythrin (PE)-conjugated mouse monoclonal antibody (mAb) against HLA-DR (Becton Dickinson, San Jose, California) or a mAb against HLA class I (W6/32) and a PE-conjugated anti-mouse IgG as second Ab. As controls, PE-conjugated IgG2a or anti-mouse IgG as second Ab were used. For intracellular staining cells were fixed in 4% paraformaldehyde in PBS and washed once with 0.1% saponin in PBS prior to staining. PBMC were double stained using fluorescein isothiocyanate (FITC)-conjugated CD3 or CD19 mAbs and a PE-conjugated antibody against HLA-DR, and single stained using PE- or FITC-conjugated antibodies against CD3, CD4, CD8, CD11b, CD11c, CD14, CD19, CD28, CD40, CD45, CD54, CD80, CD83 and CD86 (all Becton Dickinson).

Fluorescence-activated cell sorting (FACS) analysis was performed on a FACScan flow cytometer (Becton Dickinson) using Cell Quest programming.

RNA isolation and Real-Time PCR

Total RNA was isolated using the RNA-Bee extraction method (Tel-Test, Friendswood, Texas). cDNA was synthesized from 4 μg of each RNA sample using avian myeloblastoma virus reverse transcriptase (AMV-RT; Promega, Madison, Wisconsin). Expression levels of CIITA, HLA-DRA mRNA and 18S RNA were evaluated by real-time PCR using an iCycler iQ™ real-time PCR detection system and iTaq™ SYBR© Green Supermix (BioRad, Hercules California). Expression levels relative to unstimulated conditions were calculated with the comparative Ct method (relative expression level = $2^{-(\text{stimulated } \Delta Ct - \text{control } \Delta Ct)}$, ΔCt corrected for 18S expression). Sequences of used primers: CIITA forward 5′-CCGACACAGACACCATCAAC, reverse 5′-CTTTTCTGCCCAACTTCTGC; HLA-DRA forward 5′- CAAAGAAGGAGACGGTCTGG, reverse 5′-GGCTCTCTCAGTTCCACAGG.

Transient transfection

Cells were transfected with the luciferase reporter constructs pGL3-CIITA-PIV [28], pGL3-CIITA-PIIIDEL1 [29,30], pGL3-CIITA-PIII [29], pGL3-HLA-DRA [31], pGL3-HLA-B250 [32] or the pGL3-Basic luciferase reporter plasmid (Promega), in combination with the actin driven *Renilla* pGL3 reporter construct (pRL, Promega) as an internal control. HH and Raji cells were transfected by electroporation at 250 V and 960 μF (Genepulser, Bio-Rad, Hercules, California) and harvested after 48 hours. U937 cells were electroporated at 210 V and 960 μF and harvested after 24 hours. Electroporations were performed with 10^7 cells, 5 μg of pGL3 construct and 0.5 μg of actin-pRL construct (HH and Raji) or 1.25×10^7 cells, 10 μg of pGL3 construct and 1 μg of actin-pRL construct (U937). U251-MG cells were transfected with 4 μg of pGL3 construct and 0.4 μg of actin-pRL construct, using the calcium phosphate coprecipitation method [33]. After transfection, HH, Raji and U251-MG cells (which were stimulated with 500 U/ml IFN-γ) were treated with 10 μM simvastatin, or with 10 μM simvastatin and 100 μM L-mevalonate for 48 hours. U937 cells were pretreated with simvastatin for 24 hours prior to electroporation and 24 hours after electroporation. Luciferase activity was measured using the dual-luciferase reporter assay system (Promega).

Immunocytochemistry

Low density seeded HeLa cells were grown on glass coverslips in 6-well plates and stimulated with 500 U/ml IFN-γ for 72 hours with or without 10 μM simvastatin, or with 10 μM simvastatin and 100 μM L-mevalonate for 48 hours and subsequently fixed in 3% paraformaldehyde in PBS. Half of the samples were permeabilized with 0.1% TritonX -100 in PBS at 4 °C for 10 minutes. Cells were then stained for HLA-DR using mAb against HLA-DR (B8.11.2), followed by detection with the EnVision©+

System-HRP (DakoCytomation, Carpinteria, California), using an HRP-labelled polymer conjugated to a goat anti-mouse Ig. For each treatment a negative control was performed by staining with mouse IgG2b.

Isolation of lipid rafts and Western blotting

Lipid raft isolation and Western blot testing were performed according to Bouillon *et al*. [24]. Briefly, HeLa cells (10^7) were stimulated with 500 U/ml IFN-γ with or without 10 μM simvastatin, or with 10 μM simvastatin and 100 μM L-mevalonate for 48 hours. After treatment, cells were washed and lysed in 200 μl ice-cold lysis buffer containing 1% Triton X-100 for 30 minutes. Cell lysates were mixed with an equal volume of 85% sucrose (GibcoBRL, Paisley, Scotland) and transferred into TLS-55 centrifuge tubes (Beckman Coulter, Fullerton, California). Samples were overlaid with 1.2 ml 35% sucrose and 500 μl 5% sucrose and centrifuged for 16 hours at 40.000 rpm at 4°C in an Optima TLX Tabletop ultracentrifuge, using a TLS-55 rotor (Beckman Coulter). Fractions of 190 μl were collected and aliquots were mixed with sample buffer containing 5% β-mercaptoethanol and heated for 5 min at 95°C. Samples were resolved by SDS-PAGE (10 % PAA), and proteins were transferred onto Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech, Little Chalfont, England). After blocking, membranes were incubated with horseradish peroxidase (HRP)-conjugated cholera toxin B subunit (CTB-HRP, Sigma) to detect the lipid raft marker GM1, or a rabbit anti-HLA-DRα&β or a mAb against transferrin receptor (B3/25) (both from Prof. Dr. J.J. Neefjes, NKI, Amsterdam, The Netherlands) and subsequently a HRP-conjugated swine anti-rabbit or -mouse antibody (DAKO A/S, Denmark). After extensive washing the blots were subjected to chemiluminescent detection using an ECL Western blot detection kit (Amersham Bioscience).

For detection of total MHC-II protein amount total cell lysates were made of HeLa cells and PBMC. Per sample 25 ug of protein was resolved by SDS-PAGE and transferred onto Hybond ECL nitrocellulose membranes. Membranes were subsequently stained with rabbit anti-HLA- DRα&β antibody and HRP-conjugated swine anti-rabbit antibody. Membranes were stripped with 0.2 mM NaOH and stained again with mouse anti-actin antibody (Oncogene, San Diego, California) and HRP-conjugated rabbit anti-mouse antibody (DAKO) to verify equal protein loading.

Electron microscopy

For ultrastructural morphology, U937 cells were fixed in 1.5% glutaraldehyde, postfixed in 1% OsO_4 and embedded in Epon (LX-112). Ultrathin sections were stained with uranyl acetate and lead citrate. All sections were viewed with a Philips CM 10 electron microscope.

Fluorescence microscopy

Low density seeded HeLa cells were grown on glass coverslips in 6-well plates

and stimulated with 500 U/ml IFN-γ for 72 hours with or without 10 μM simvastatin, or with 10 μM simvastatin and 100 μM L-mevalonate for 48 hours, fixed in 3.7% paraformaldehyde in PBS, washed, stained with a rabbit anti-HLA-DRα&β antibody and a Texas Red conjugated goat anti-rabbit antibody (Molecular Probes, Leiden The Netherlands) and subsequently with Alexa-488 conjugated cholera toxin B subunit (Molecular Probes) to detect the lipid raft marker GM1. Confocal Images were collected with a Leica TCS SP2 system (Leica Microsystems, Heidelberg, Germany) equipped with an Argon/Krypton laser. For dual analyses green fluorescence was detected at λ=520-560 nm after excitation with λ=488 nm and red fluorescence was detected at λ>585 nm after excitation with λ=568nm. Serial sections were made to minimize possible leak through of the different fluorochromes during imaging.

Results

Inhibition of MHC-II and MHC-I cell surface expression by statins

We have studied the effect of simvastatin on cell surface expression of MHC-II molecules in several cell types. The concentrations of simvastatin applied in our *in vitro* assay range from 1 to 10 μM, which overlaps in part therapeutical concentration ranges. We found that simvastatin effectively represses IFN-γ induction of MHC-II cell surface expression in a dose dependent manner in the monocytic cell lines U937 (Figure 1A) and THP-1 (not shown), as determined by FACS analysis. L-mevalonate reverses the effect of simvastatin (Figure 1B), which indicates that this repression is indeed mediated through inhibition of HMG-CoA reductase. A similar inhibition on MHC-II expression induced by IFN-γ is also noted in HeLa cells (cervical carcinoma, Figure 1C) and the glioma cell lines U251-MG and U373-MG (not shown), which indicates that the effect of simvastatin is not confined to cells of the immune system, but rather reflects a general effect on the induction of MHC-II membrane expression by IFN-γ. Depending on the cell line tested, the reduction in IFN-γ-induced MHC-II expression by simvastatin varies from 3 (U937) to 10 (HeLa) fold. Similar results were obtained with the statins lovastatin and atorvastatin (not shown). Notably, simvastatin is also able to completely inhibit the IFN-γ induction of MHC-I on HeLa cells (Figure 1D). Furthermore, besides inhibiting IFN-γ induction of MHC-I expression, simvastatin also lowers constitutive cell surface expression of MHC-I on HeLa cells (4 to 5 fold; Figure 1E). This illustrates that the inhibitory effect of simvastatin on cell surface expression is not confined to MHC-II molecules, but affects both classes of antigen presentation molecules. Simvastatin treatment does not induce significant numbers of apoptotic or necrotic cells in HeLa cells, as evaluated by eosin staining and DNA fragmentation analysis through propidium iodide staining (data not shown). This indicates that the effect of simvastatin on cell surface expression of MHC molecules is not due to cytotoxic effects of statins.

We further investigated whether the repression of MHC-II surface expression by simvastatin was specific for the IFN-γ-inducible expression of MHC-II or whether constitutive expression of MHC-II molecules as observed in B lymphocytes or MHC-II expression as observed in activated human T lymphocytes would also be affected by simvastatin. In primary peripheral blood mononuclear cells (PBMC), simvastatin reduces cell surface expression of MHC-II on both activated T lymphocytes (CD3

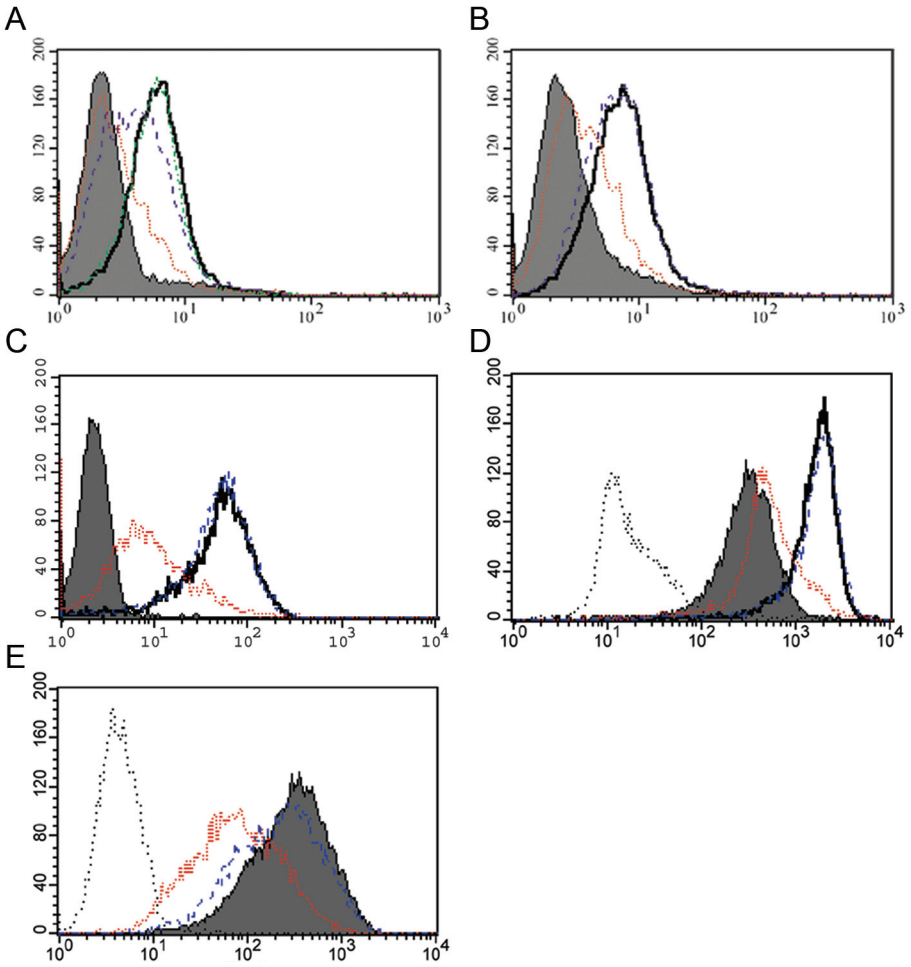

Figure 1. Statins downregulate MHC-II and MHC-I cell surface expression. Flow cytometric analysis for MHC-II (A to C) and MHC-I (D and E) cell surface expression. **A** MHC-II cell surface expression on U937 cells treated with IFN-γ (500 U/ml, 48 hours) alone (thick black line) or in combination with 1 μM (green dotted-striped line), 5 μM (blue striped line) or 10 μM (red dotted line) simvastatin. **B** MHC-II cell surface expression on U937 cells treated with IFN-γ alone (thick black line) or in combination with simvastatin (10 μM, red dotted line), or simvastatin and L-mevalonate (100 μM, blue striped line). **C** and **D** MHC-II (C) and MHC-I (D) cell surface expression on HeLa cells treated with IFN-γ (500 U/ml, 72 hours) alone (thick black line) or in combination with simvastatin (10 μM, 48 hours, red dotted line), or simvastatin and L-mevalonate (100 μM, 48 hours, blue striped line). **D** Constitutive expression of MHC-I is also indicated (solid histogram). **E** Constitutive MHC-I cell surface expression on HeLa cells treated with simvastatin (10 μM, 48 hours, red dotted line) alone or in combination with L-mevalonate (100 μM, 48 hours, blue striped line). Each panel is a histogram representing cell numbers (y-axis) versus log fluorescence intensity (x-axis). For all panels solid histograms represent MHC-II and MHC-I cell surface expression under unstimulated conditions. A to C Isotype control staining did not differ significantly between unstimulated or stimulated conditions and was comparable to MHC-II staining under control conditions. D and E Dotted black line depicts control fluorescence of secondary antibody only. A to E are representative of at least three independent experiments.

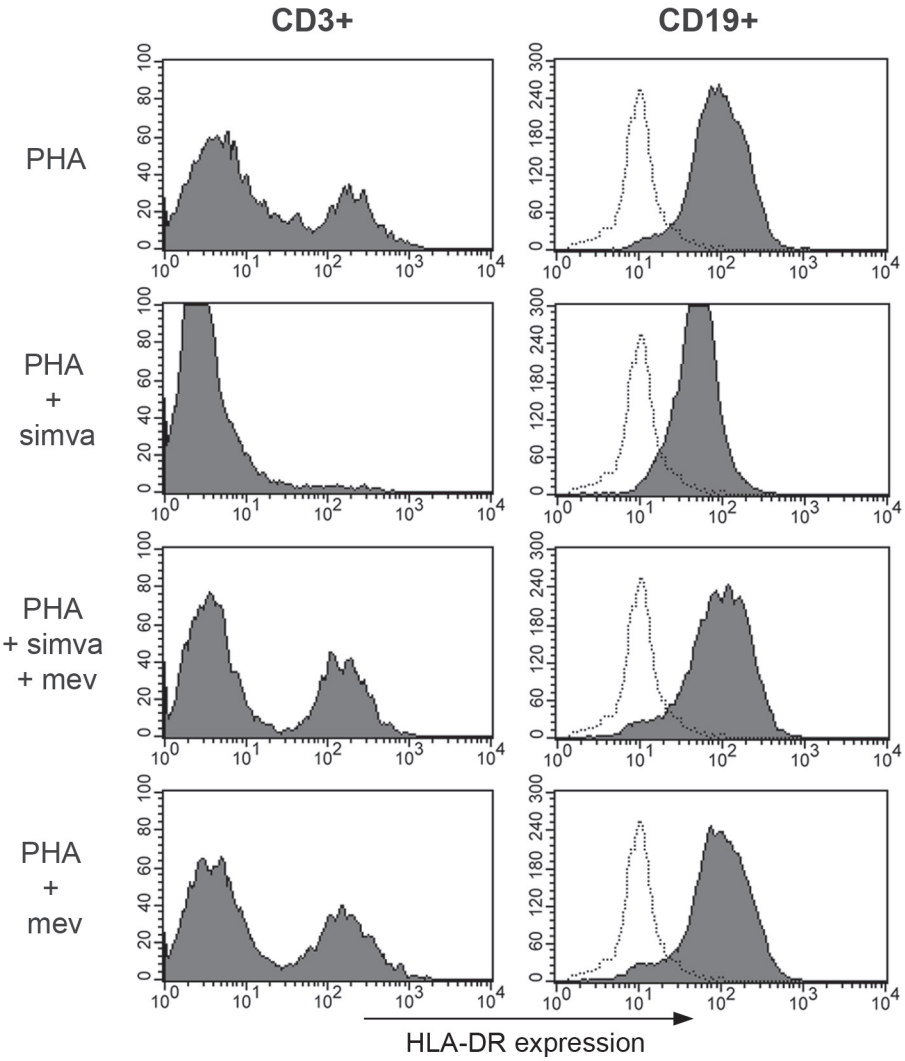

Figure 2. Simvastatin downregulates MHC-II cell surface expression in human activated T and B cells. Flow cytometric analysis for MHC-II cell surface expression on human T lymphocytes (CD3 positive) and B lymphocytes (CD19 positive) treated with PHA (1 µg/ml, 3 days) alone or in combination with simvastatin (10 µM, simva), simvastatin and L-mevalonate (100 µM, mev) or L-mevalonate. Solid histograms represent the relative intensity of HLA-DR staining (x-axis) of cells gated for CD3 or CD19 positivity. Dotted lines depict isotype control staining. Shown is a representative of independent experiments with PBMC obtained from 5 different donors.

positive) and B lymphocytes (CD19 positive) (Figure 2). Notably, hardly any MHC-II expressing T lymphocytes can be observed after simvastatin treatment. In contrast, MHC-II expression on B lymphocytes is slightly decreased by simvastatin (2 fold). L-mevalonate again reverses the effect of simvastatin, whereas treatment with L-mevalonate alone does not affect cell surface expression of MHC-II (Figure 2).

MHC-II transcription, promoter activation, and translation

Subsequently we investigated the effect of simvastatin on IFN-γ-inducible transcript levels with emphasis on HLA-DRA and the class II transactivator (CIITA), the

masterswitch for MHC-II expression [34], in HeLa cells by real-time RT-PCR. Figure 3A shows that simvastatin treatment did not reduce the levels of either CIITA or HLA-DRA mRNA induced by IFN-γ in HeLa cells. In addition, simvastatin did not affect CIITA and HLA-DRA transcript levels in activated T cells (not shown, [35]). Expression of *MHC2TA* is controlled by the usage of four different *MHC2TA* promoters (CIITA-P) [34]. Previously, we have shown that CIITA-PIII is employed by activated T cells and by B cells [29,36], whereas CIITA-PIV and also CIITA-PIII, through its IFN-γ regulatory region (DEL1), are utilized following activation by IFN-γ in a variety of different cell types [29,30]. In luciferase-reporter assays, simvastatin was unable to inhibit the activity of either *MHC2TA* or *HLA-DRA* promoters in various cell lines tested (Figure 3B-E). Rather, simvastatin treatment resulted in an increase in the activity of these promoters, an effect that was reversed by the presence of L-mevalonate. This is in line with the elevations in mRNA levels for CIITA and HLA-DRA observed after simvastatin treatment (Figure 3A). In addition, the same effect was observed with a construct containing the *HLA-B* promoter (not shown), which could reveal a generally enhanced transcriptional activity due to simvastatin treatment. Next, we investigated the effect of simvastatin on the total cellular amount of MHC-II proteins in PBMC and HeLa cells. For this, we obtained total cell lysates of simvastatin treated cells and investigated MHC-II protein content by Western Blot. Figure 3 shows that simvastatin treatment results in a minimal reduction of the total amount of MHC-II proteins in PBMC (Figure 3F) or in IFN-γ stimulated HeLa cells (Figure 3G). Together, this infers that simvastatin does not significantly inhibit the transcription/translation pathway of MHC-II.

Transport of MHC-II molecules to the cell surface

Because simvastatin does reduce the IFN-γ-induced MHC-II molecule expression on the cell membrane of HeLa cells (Figure 1C), but not the cellular amount of MHC-II protein (Figure 3G), we hypothesized that simvastatin does not inhibit the synthesis of MHC-II molecules, but their transport to the cell surface. Therefore, we investigated whether simvastatin impairs MHC-II transport to the cell membrane by staining HeLa cells with and without permeabilizing the cells. When the cells are not permeabilized, IFN-γ treated HeLa cells display a normal membrane staining for MHC-II, covering the nucleus, whereas on cells co-treated with simvastatin hardly any membrane staining can be observed (Figure 4A; membrane staining). Some simvastatin treated cells stain positive for MHC-II, however this staining seems to be intracellular and could be due to some degree of permeabilization caused by the simvastatin treatment or the fixation protocol (IFN-γ-treated cells also display a similar spotted staining). L-mevalonate restores normal MHC-II staining, similar to staining after IFN-γ treatment (not shown). However, when intracellular MHC-II is stained, the same pattern of staining can be observed in both IFN-γ-treated and simvastatin-treated cells, indicating that MHC-II molecules are still present in simvastatin-treated cells (Figure 4A; intracellular staining). To quantify the amount of intracellular MHC-II in simvastatin-treated HeLa cells, we

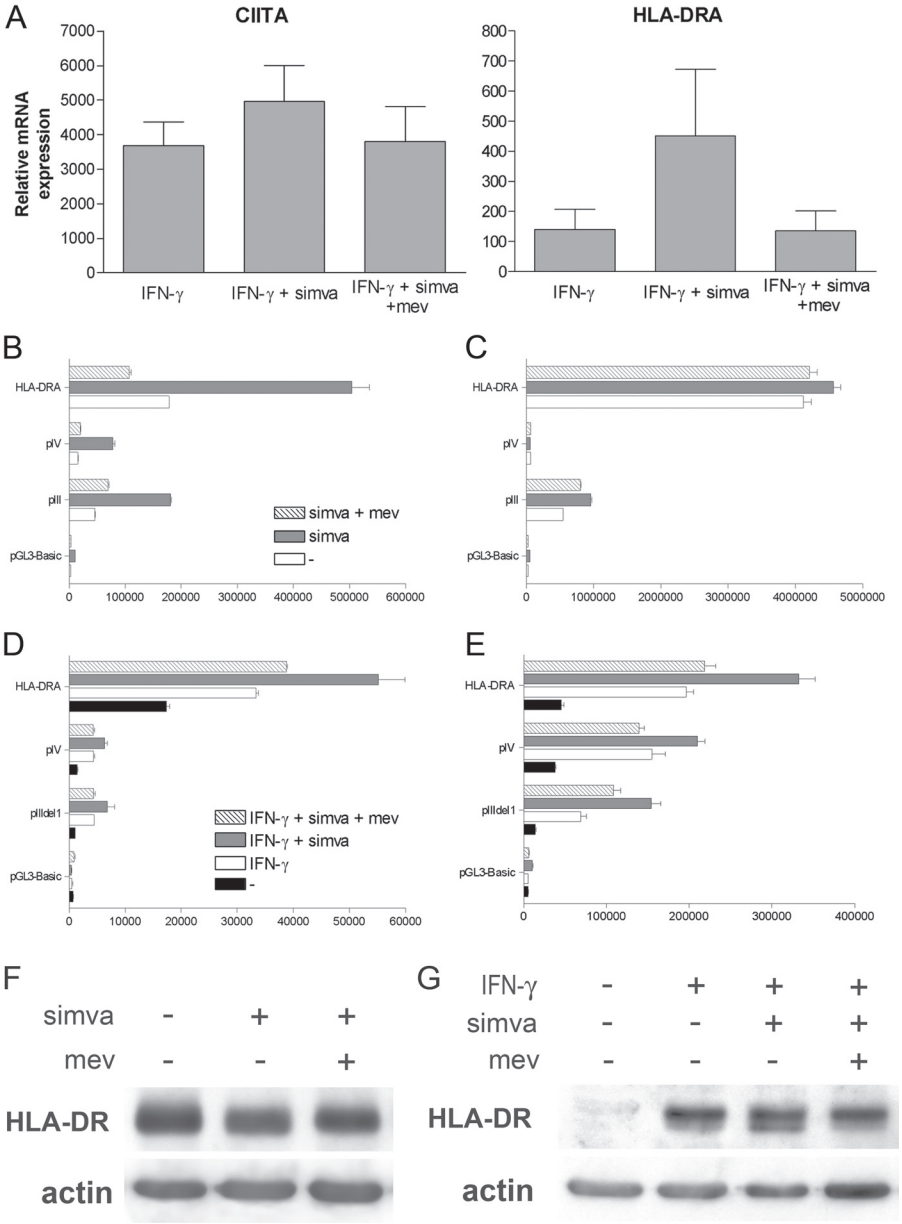

Figure 3. Simvastatin does not affect transcription of the MHC2TA or HLA-DRA genes nor total MHC-II protein amount. **A** Real Time PCR analysis of CIITA and HLA-DRA mRNA levels in HeLa cells treated with IFN-γ alone (500 U/ml, 12 hours) or in combination with simvastatin (10 μM, simva) or simvastatin and L-mevalonate (100 μM, mev). Depicted are expression levels relative to unstimulated conditions calculated with the comparative Ct method (comparative expression level = $2^{-(\text{stimulated } \Delta Ct - \text{control } \Delta Ct)}$, ΔCt corrected for 18S expression). **B** to **E**, Transient promoter-reporter assays for the HLA-DRA, CIITA-PIII and CIITA-PIV promoters. **B** HH cells and **C** Raji cells transfected with luciferase-reporter constructs containing the CIITA-PIII, CIITA-PIV or HLA-DRA promoters or the pGL3-Basic luciferase-reporter plasmid as a control, treated with simvastatin (10 μM, 48 hours, simva) alone or in combination with L-mevalonate (100 μM, mev). **D** U937 cells and **E** U251-MG cells transfected with luciferase-reporter constructs containing the CIITA-PIIIDEL1, CIITA-PIV or HLA-DRA promoters or the pGL3-Basic luciferase reporter plasmid as a control, treated with IFN-γ (500 U/ml, 48 hours) alone or in combination with simvastatin (10 μM) or simvastatin and L-mevalonate (100 μM). B to E Depicted are relative light units (RLU). A through E, depicted are mean + sem of one representative of three independent experiments. **F** and **G** Western Blot analysis of total MHC-II protein amount in (F) PBMC following simvastatin treatment (10 μM, 3 days, simva) alone or in combination with L-mevalonate (100 μM, mev) and (G) HeLa cells treated with IFN-γ (500 U/ml, 48 hours) alone or in combination with simvastatin (10 μM, simva) or simvastatin and L-mevalonate (100 μM, mev). Restaining of blots for actin verified equal protein loading. F and G are representative of three independent experiments.

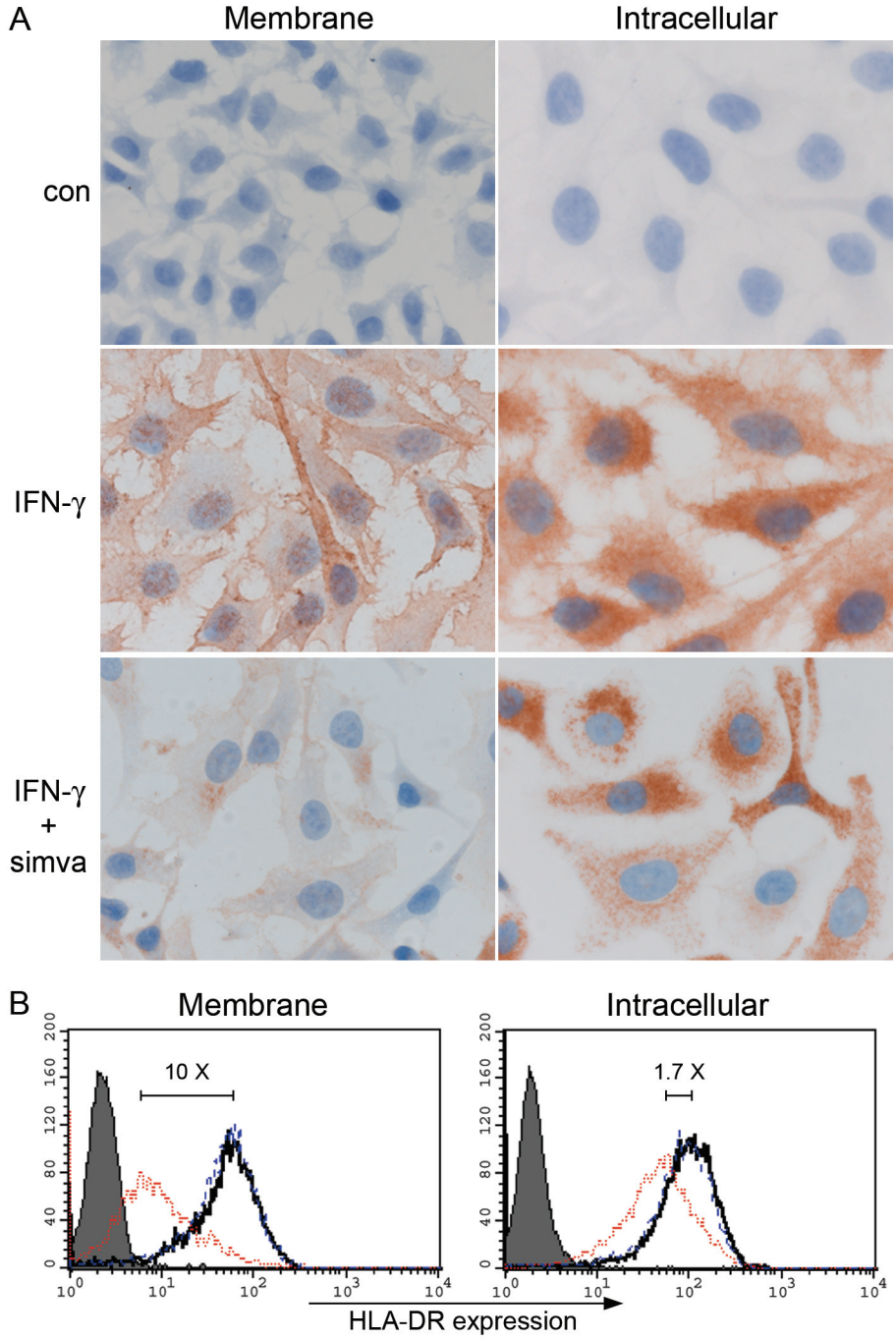

Figure 4. Simvastatin impairs MHC-II transport to the cell surface. **A** Immunocytochemical analysis of membrane and intracellular HLA-DR protein expression in HeLa cells treated with IFN-γ (500 U/ml, 72 hours) alone or in combination with simvastatin (10 μM, 48 hours, simva), or simvastatin and L-mevalonate (100 μM, 48 hours, mev). Isotype control staining was negative for all treatments. Shown is a representative of four independent experiments. **B** Flow cytometric analysis for MHC-II cell surface (membrane) and intracellular expression in HeLa cells treated with IFN-γ (500 U/ml, 72 hours) alone (thick black line) or in combination with simvastatin (10 μM, 48 hours, red dotted line), or simvastatin and L-mevalonate (100 μM, 48 hours, blue striped line). Solid histograms represent MHC-II cell surface expression under unstimulated conditions. Shown is a representative of three independent experiments.

analyzed intracellular expression as well as cell surface expression of MHC-II by FACS staining. Figure 4B shows that, whereas simvastatin reduces the membrane expression of MHC-II about 10 fold, intracellular MHC-II expression is only slightly reduced by simvastatin treatment (1.7 fold). Therefore, it seems that the effect of simvastatin on MHC-II cell surface expression is caused mainly by impairment of transport of MHC-II molecules to the cell surface.

Intracellular distribution of MHC-II molecules following simvastatin treatment

Next, we further examined the effect of simvastatin treatment on the cellular distribution of MHC-II. Because cholesterol, the major end product of the mevalonate pathway, is one of the main components of lipid rafts [14], we studied the effect of simvastatin on lipid raft integrity and association of MHC-II molecules with lipid rafts. These lipid rafts are involved in transport and concentration of MHC-II molecules to the cell surface [20-24]. For those purposes, HeLa cells were treated with IFN-γ to induce MHC-II cell surface expression. Next, these MHC-II expressing HeLa cells were lysed and submitted to sucrose gradient fractionation for lipid raft analysis. The results of these fractionation experiments show that the intracellular distribution of MHC-II molecules is altered by simvastatin treatment (Figure 5, HLA-DR). Raft and non-raft fractions are visualized by staining the blot with HRP-conjugated cholera toxin B subunit, which binds to ganglioside GM-1, and mAb against the transferrin receptor, which is known to be located in the non-raft compartment of the plasma membrane [17,37,38]. In this experimental setup, lipid rafts transfer to the low weight fractions around fractions 2 through 5 during ultracentrifugation. Under IFN-γ-induced conditions, MHC-II is found partly in the lipid raft fractions and partly in the non-raft fractions (Figure 5, HLA-DR). After simvastatin treatment, the integrity of lipid rafts is compromised, illustrated by the GM-1 pattern, which now seems to be condensed to one major fraction (Figure 5, GM-1). This observed disruption of lipid rafts coincides with loss of association of MHC-II molecules with these intracellular vesicles (in the low-weight fractions). Normal lipid raft and MHC-II distribution is restored by L-mevalonate (not shown). The same effect is observed in U251-MG cells (not shown). The distribution of the non-raft resident transferrin receptor is not altered by simvastatin treatment, revealing that simvastatin only affects the distribution of raft-resident molecules (Figure 5, TFR).

Intracellular vesicle degradation

We further investigated the effect of simvastatin on the presence and localization of cholesterol-containing intracellular vesicles by ultra-structural morphology electron microscopy (EM). Exposure to simvastatin after IFN-γ treatment has a dramatic effect on the overall integrity of U937 cells and results in an almost complete absence of intracellular vesicles, including mitochondria (Figure 6, compare panel C with panels A, B and D). In addition, the abundant Golgi stacks that are present in

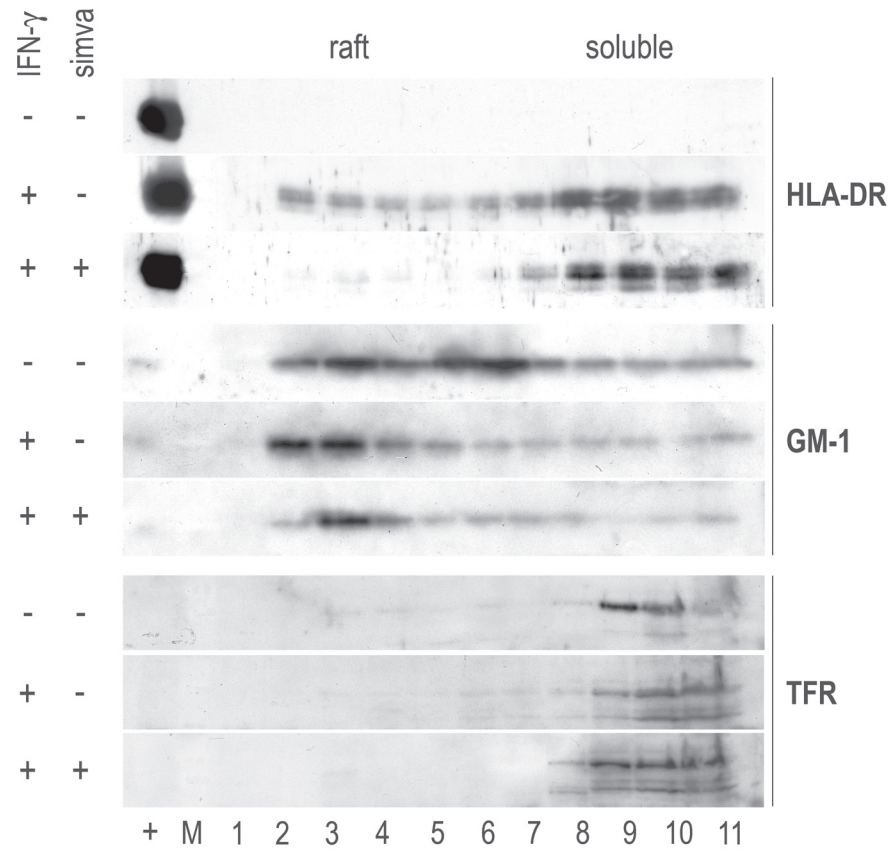

Figure 5. Simvastatin disrupts MHC-II containing lipid rafts. Lipid raft distribution after sucrose gradient fractionation of unstimulated HeLa cells, Hela cells treated with IFN-γ (500 U/ml, 48 hours) alone or in combination with simvastatin (10 μM, simva). Blots stained with rabbit anti-HLA-DRA&B antibody and restained with cholera toxin B subunit, staining the lipid raft marker GM1, and a mouse anti-transferrin receptor (TFR) antibody. Fractions are numbered from low-weight (1) to high-weight (11), +: positive control (Raji B cell lysate), M: molecular weight marker. Representative of three independent experiments.

untreated or IFN-γ-treated cells also disappear after simvastatin treatment (Figure 6A,B,D, asterisks). The clusters of large vacuolar structures we frequently observed in simvastatin treated cells might represent remnants of the Golgi apparatus (Figure 6C, arrow). Furthermore, we noted a disruption in the localization and shape of the nucleus in these simvastatin treated U937 cells (Figure 6, compare inserts in panels A and C). Addition of L-mevalonate completely reverses the effect of simvastatin and results in the reappearance of these intracellular vesicles and normal Golgi stacks in the cytoplasm, and a normal localization and shape of the nucleus (Figure 6D; and data not shown).

Localization of MHC-II in lipid rafts

To finally determine the localization of MHC-II molecules in lipid rafts, we analyzed the distribution of MHC-II and the lipid raft marker GM-1 on HeLa cells by confocal fluorescence microscopy. Following IFN-γ treatment, HeLa cells display a

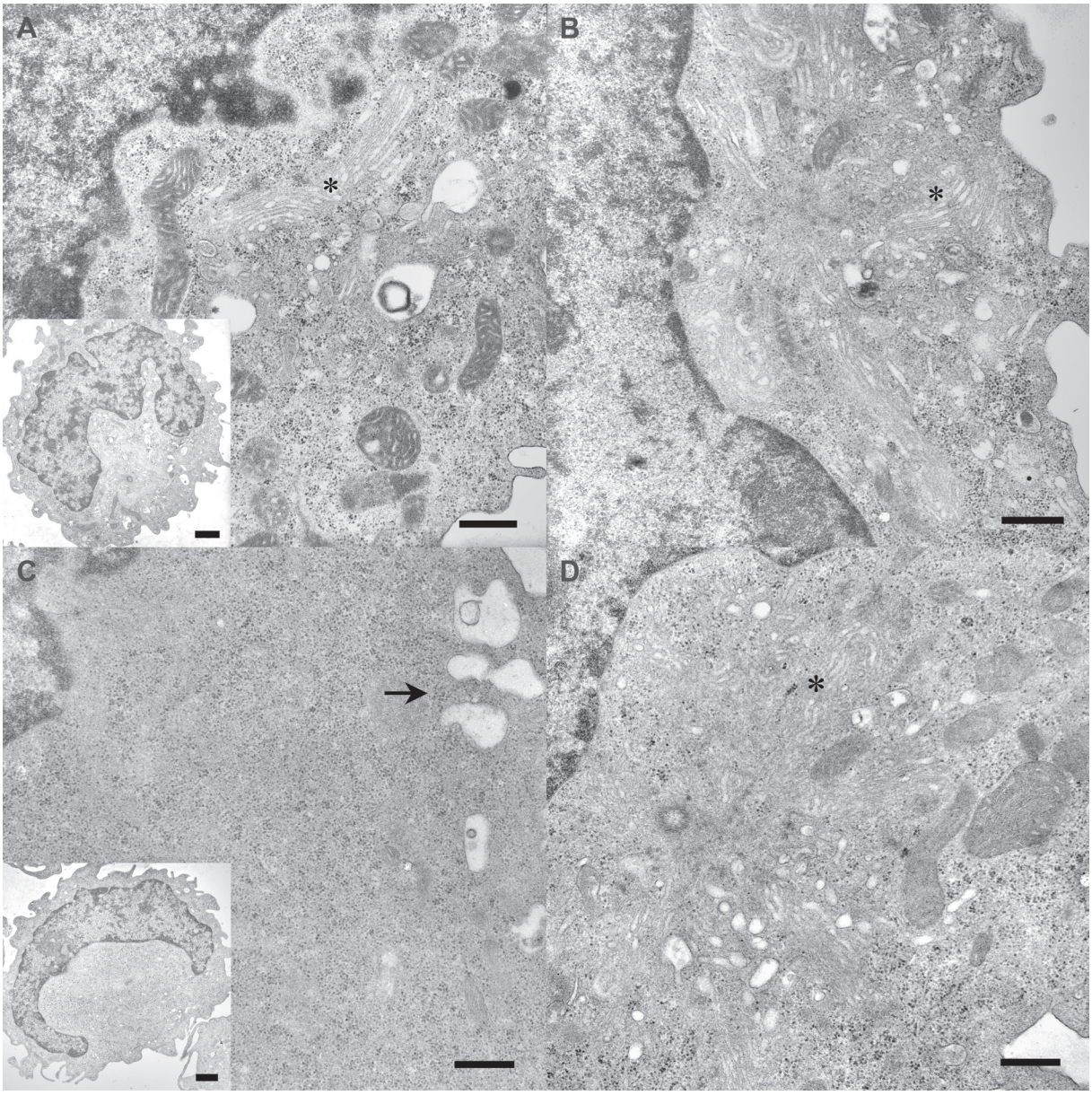

Figure 6. Simvastatin induces intracellular vesicle degradation. Presence of intracellular vesicles visualized by electron microscopy. **A** Unstimulated U937 cells. **B** U937 cells treated with IFN-γ (500 U/ml, 48 hours) alone or (**C**) in combination with simvastatin (10 μM) or (**D**) simvastatin and L-mevalonate (100 μM). Note the abundant Golgi stacks in A, B and D (asterisk) and a cluster of vacuoles in C (arrow). Inserts in A and C show the altered overall structure and localization of the nucleus in simvastatin treated U937 (C) as compared to untreated cells (A). Bars: A to D: 500 nm, inserts in A and C: 1000 nm.

highly clustered distribution of MHC-II molecules intracellularly as well as membrane expression of MHC-II (Figure 7). Both clustered MHC-II expression and MHC-II membrane expression are colocalized to a high degree with GM-1-containing patches. Simvastatin treatment clearly reduces the membrane expression of both MHC-II and GM-1, whereas intracellular MHC-II and GM-1 are still present in condensed sphere-like structures. It is noted that the remaining MHC-II expression seems to be confined to the outer edge of these structures, whereas GM-1 is only present in the center (Figure

7, additional observations). These structures therefore might represent remnants of MHC-II containing intracellular vesicles, which correspond to the vacuolar structures also observed in EM.

Reduction in cell surface expression of additional immunoregulatory molecules on human primary lymphocytes by simvastatin

In addition to investigating the effect of simvastatin treatment on MHC-II and MHC-I membrane expression, we also examined the effect of simvastatin on several other immunoregulatory molecules, which are important for full immune activation. We determined the membrane expression of these molecules on freshly isolated PBMC prior to and after simvastatin treatment. We found that simvastatin treatment significantly reduces the membrane expression of a variety of molecules on PBMC (Table I). Simvastatin reduces membrane expression of CD3, CD4 and CD8, T-cell co-receptors essential for TCR / MHC-II or MHC-I-peptide complex interactions and signaling, as well as the B cell co-receptor CD19. Simvastatin treatment also results

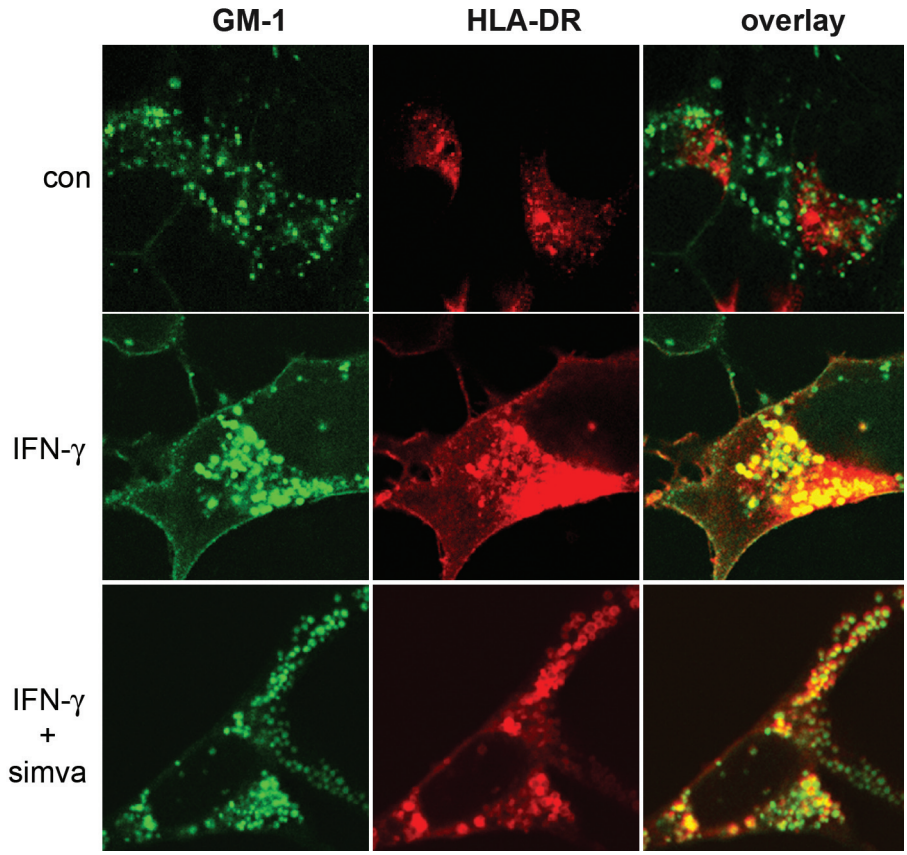

Figure 7. MHC-II is localized in lipid rafts, which are destroyed by simvastatin, visualized by confocal fluorescence microscopy. HeLa cells treated with IFN-γ (500 U/ml, 72 hours) alone or in combination with simvastatin (10 µM, 48 hours) or simvastatin and L-mevalonate (100 µM, 48 hours) and stained with cholera toxin B subunit staining the lipid raft marker GM1 (green, left panels) and anti-HLA-DRA&B (red, middle panels). The overlay of the two images is shown in the right panel. One representative of two independent experiments is shown.

in lowered expression of the co-stimulatory molecules CD28, expressed by T cells, and CD40, CD80 and CD86, expressed by B cells and other antigen presenting cells (APC). In addition, simvastatin lowers the expression of the integrins CD11b and CD11c and the LPS receptor CD14, which are expressed mostly by monocytes. Finally, simvastatin also decreases the membrane expression of the dendritic cell (DC) marker CD83 and the commonly expressed adhesion molecule CD54 (ICAM-1). L-mevalonate is able to restore the membrane expression of all of these molecules to normal levels. This shows that simvastatin does not only affect MHC-II and MHC-I expression, but that it also interferes with the expression of several other molecules important for full immune activation. Markedly, although the expression of most molecules is affected by simvastatin, simvastatin treatment does not alter the expression of the common leukocyte antigen CD45, a molecule that is excluded from lipid rafts [39].

Discussion

In this report, we show that treatment of cells with simvastatin results in down regulation of cell surface expression of MHC-II molecules on a variety of cell types. Simvastatin not only affects IFN-γ-induced MHC-II expression, as has been observed before [11-13], but also constitutive expression of MHC-II on activated T- and B lymphocytes [13]. In our evaluation with respect to the mechanism lying behind this downregulatory effect we have determined that simvastatin does not affect the transcription of the *MHC2TA* gene, as suggested previously [11,12,35] and only minimally affects the intracellular pool of MHC-II protein. Therefore we hypothesize that simvastatin could affect the transport of MHC-II molecules to the cell surface. This is supported by our observation that simvastatin treatment disrupts the association of MHC-II molecules with cholesterol-containing microdomains, or lipid rafts. Because a large portion of MHC-II molecules is localized in lipid rafts [20-24,40,41], disruption of MHC-II-containing lipid rafts and resulting impairment of transport of MHC-II molecules to the cell surface effectively reduces the amount of cell surface expressed MHC-II molecules.

The discrepancy we observe in the effect of simvastatin on transcriptional activity and protein expression still raises some questions. First, an increase in both CIITA and HLA-DR mRNA levels and promoter activity is observed as a result of simvastatin treatment. Because this increase in activity seems to result from a general effect of simvastatin, this enhanced transcriptional activity could be due to a higher availability of transcription factors and their accessibility to promoter elements caused by disruption or enhanced permeability of the nuclear membrane. Second, this enhanced transcription does not result in enhanced MHC-II protein expression. Indeed, a slight reduction in protein level can be observed after simvastatin treatment. Therefore it seems that, despite an increase in transcription, MHC-II protein synthesis is not enhanced by simvastatin, which could possibly be due to a synthesis impairment. An alternative explanation could be that the increased transcription does result in a

Table 1. Simvastatin affects membrane expression of various immunoregulatory molecules on human PBMC.

Cell type	Marker	Function	Postive cells (PBMC)[a] (%)		
			con	simva	simva + mev
T-cell	CD3	TCR signalling complex	44.4 ± 0.8	32.5** ± 2.1	41.2 ± 0.9
	CD4	MHC-II receptor	33.2 ± 0.8	25.0* ± 1.9	30.1† ± 0.5
	CD8	MHC-I receptor	22.5 ± 2.0	14.6* ± 1.3	19.7† ± 1.6
	CD28	costimulation	45.1 ± 1.3	34.4* ± 2.3	42.2* ± 1.4
B-cell	CD19	B-cell coreceptor subunit	6.2 ± 0.2	4.1** ± 0.4	5.9* ± 0.3
	CD40	costimulation	6.5 ± 0.6	4.0† ± 0.8	5.5 ± 1.0
	CD80	costimulation	14.8 ± 0.9	5.8** ± 0.7	14.1** ± 1.0
	CD86	costimulation	20.1 ± 0.4	11.8* ± 2.2	18.4* ± 0.2
Monocyte	CD11b	adhesion	21.9 ± 0.6	18.8† ± 0.0	23.8** ± 0.3
	CD11c	adhesion	25.04 ± 2.0	15.1** ± 1.1	20.5** ± 1.0
	CD14	LPS-binding protein receptor	14.5 ± 1.2	1.0* ± 0.0	11.9* ± 1.0
DC	CD83	-	17.9 ± 1.8	5.9** ± 1.0	13.2† ± 2.9
Leukocytes	CD54	adhesion	60.3 ± 0.9	48.2** ± 2.4	58.5* ± 2.7
	CD45	Leukocyte Common Antigen	79.2 ± 2.8	78.3 ± 3.6	77.1 ± 3.0

[a] Flow cytometric analysis for cell surface expression on human total PBMC treated with PHA (1 µg/ml, 3 days) alone (con) or in combination with simvastatin (10 µM, simva) or simvastatin and L-mevalonate (100 µM, mev). Depicted are mean percentages ± sem of independent experiments with PBMC from 4 different donors. Statistically significant differences between control cells and simvastatin treated cells (simva), and simvastatin treated cells and simvastatin and L-mevalonate-treated cells (simva + mev), according to two-tailed student's T test are depicted: † $P < 0.1$, * $P < 0.05$, ** $P < 0.01$.

higher rate of MHC-II protein synthesis, but protein breakdown is enhanced because these proteins cannot be incorporated into lipid rafts. Altogether it is clear that further research on the kinetics of MHC-II molecule synthesis and breakdown following statin treatment is needed to elucidate these issues. In this respect, differences in MHC-II kinetics could also account for differences in effect of simvastatin between cell types. For instance, on B lymphocytes simvastatin induces only a small decrease in MHC-II expression, whereas on T lymphocytes MHC-II expression is completely blocked. This could be due to the fact that MHC-II expression on B lymphocytes is very stable, with a low turnover, whereas in activated T cells MHC-II is actively transported to the cell surface by newly formed lipid rafts.

Glycosylphosphatidylinositol (GPI)-linked proteins are in general characteristic components of biochemically defined lipid rafts and rely for transport, recycling and function at the cell surface on the integrity of these cholesterol-containing vesicles [14]. Therefore, disruption of lipid rafts does not only affect cell surface expression of MHC-II molecules. Indeed, we show that on human PBMC simvastatin significantly reduces

the expression of a variety of immunologically relevant molecules, which include, besides MHC-II and MHC-I, also CD3, CD4, CD8, CD11b, CD11c, CD14, CD19, CD28, CD40, CD80, CD86 and CD54. These molecules have all been shown to be incorporated in lipid rafts [18,20-24,39-47]. This is in contrast to CD45, a molecule that does not associate with lipid rafts [39], the cell surface expression of which is not affected by simvastatin treatment.

Based on our current findings we postulate that simvastatin or statins in general could have a much broader impact on immune system function. Statins are potent inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) and thereby affect the mevalonate pathway, which is important for the biosynthesis of isoprenoids such as geranylgeranylpyrophosphate (GGPP) and farnesylpyrophosphate (FPP), as well as cholesterol [1]. Notably cholesterol-containg microdomains, or lipid rafts, are a key element of organization essential for signaling between immune cells and immune cell activation [39]. Furthermore, GGPP and FPP are important lipid attachments for the post-translational modification of several proteins that include the small GTP-binding proteins Ras, Rac, and Rho. Attachment of these lipids, so-called isoprenylation, is essential for activation and intracellular transport of proteins crucial for various cellular functions, such as maintenance of cell shape, motility, factor secretion, differentiation, and proliferation [48,49]. Therefore, it seems plausible that statins do not only affect the expression of MHC-II, but rather have a general effect on lipid raft-mediated intracellular transport and cell surface expression of a variety of molecules or on the activation of certain proteins by isoprenylation, thereby affecting multiple cellular functions.

In this respect, recognition of MHC-II- or MHC-I-peptide complexes by respectively antigen-specific $CD4^+$ or $CD8^+$ T cells requires accumulation of antigen presenting molecules in membrane microdomains at the APC, which act as signaling platforms for MHC-peptide/TCR interactions in the immunological synapse, resulting in efficient T cell activation [18,21-25]. Because we show that simvastatin hampers the formation of lipid rafts, and so the transport of intra-cellular produced MHC-II molecules to the cell membrane and their sustained expression on the cell membrane, this would infer that simvastatin treatment results in impairment of immunological synapse formation during TCR/peptide-MHC interactions at the T cell-APC interface. Indeed, it has been shown that lipid raft disruption in APC alters the interaction between APC and T lymphocytes [22,40,41].

Likewise, on the T cell side of the immunological synapse, T cell rafts contain amongst others the TCR-CD3 complex, a portion of the CD4 and CD8 co-receptors and a number of components of the T cell signalosome [39,42,50]. Furthermore, following T cell engagement expression of co-stimulatory molecules such as CD40, CD80 and CD86 on APC and CD28 on T cells and adhesion molecules such as CD54 (ICAM-1) in lipid rafts is essential for immunological synapse formation and optimal immune activation [23,39,43-47]. We show that simvastatin significantly reduces the expression of

these molecules at the cell surface of freshly isolated human PBMC. In addition, not only immune receptors and co-receptors are often organized in lipid rafts. Many signaling components essential for signal transduction into the cell are raft-associated [50,51]. Therefore, we hypothesize that disruption of lipid rafts by statins could impair not only critical functions in antigen presentation and immune activation provided by the APC, but could also affect T cell-associated functions essential for activation, differentiation and functioning of $CD4^+$ and $CD8^+$ T cells. This is corroborated by observations that statin treatment reduces the ability of antigen presenting cells to present antigens to and activate T cells and the ability of T cells to proliferate upon activation [12,13]. In addition, we show that simvastatin decreases the membrane expression of monocyte-expressed integrins CD11b and CD11c and the LPS receptor CD14, which have shown to be expressed in lipid rafts [52,53]. This implies that simvastatin treatment might also result in impairment of monocyte activation and function.

On the basis of the results of our studies we propose that the main mechanism that accounts for lowered cell surface expression of MHC-II and other immunologically relevant molecules by simvastatin treatment is disruption of cholesterol-containing microdomains, or lipid rafts. Moreover, because lipid rafts fulfill additional essential cellular functions, disruption of lipid rafts affects a multitude of cellular processes not only critical for antigen presentation and resulting immune activation but also for a variety of other cell signaling and cell survival processes.

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