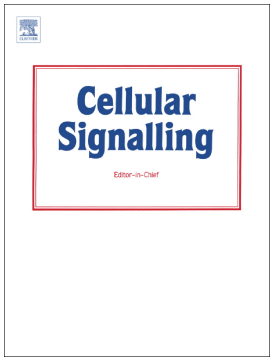



# The HLA-DR mediated signalling increases the migration and invasion of melanoma cells, the expression and lipid raft recruitment of adhesion receptors, PD-L1 and signal transduction proteins

Francesca Costantini, Giovanna Barbieri

PII: S0898-6568(17)30137-7
DOI: doi: 10.1016/j.cellsig.2017.05.008
Reference: CLS 8920

To appear in: *Cellular Signalling*

Received date: 14 February 2017
Revised date: 25 April 2017
Accepted date: 6 May 2017

Please cite this article as: Francesca Costantini, Giovanna Barbieri , The HLA-DR mediated signalling increases the migration and invasion of melanoma cells, the expression and lipid raft recruitment of adhesion receptors, PD-L1 and signal transduction proteins, *Cellular Signalling* (2017), doi: 10.1016/j.cellsig.2017.05.008

# The HLA-DR mediated signalling increases the migration and invasion of melanoma cells, the expression and lipid raft recruitment of adhesion receptors, PD-L1 and signal transduction proteins

Francesca Costantini[1] and Giovanna Barbieri[1*]

1. Istituto di Biomedicina e Immunologia Molecolare "Alberto Monroy",

Consiglio Nazionale delle Ricerche (CNR),

Via Ugo La Malfa 153,

90146 Palermo, Italy.

Francesca Costantini e-mail: francesca.costantini@ibim.cnr.it

* Corresponding author:

Giovanna Barbieri, PhD;

Istituto di Biomedicina e Immunologia Molecolare "Alberto Monroy",

Consiglio Nazionale delle Ricerche (CNR),

Via Ugo La Malfa 153,

90146 Palermo, Italy.

Fax n°: +390916809548

Tel n°: +390916809537/29

e-mail: barbieri@ibim.cnr.it

## Abstract

The constitutive expression of Major Histocompatibility Complex (MHC) class II molecules is restricted to professional Antigen-Presenting Cells (APCs), nevertheless almost 50% of melanomas express constitutively the MHC class II molecules. Therefore, in two MHC class II constitutive expressing melanoma cell lines we studied the signalling mediated by the HLA-DR molecules in the aim to understand the consequence of class II mediated signalling on metastatic dissemination of melanoma. In particular, we reported that the HLA-DR mediated signalling play a new role in melanoma progression, increasing the migration and invasion of melanoma cells. Furthermore, we showed that the HLA-DR mediated signalling increases the expression and the lipid raft localisation of class II molecules, PD-L1 receptor, Integrin and CAM adhesion receptors, FAK, AKT and STAT3 signalling proteins. We also showed that the HLA-DR mediated signalling increases the activation of FAK, AKT, ERK, PKC and STAT3 signalling proteins and the expression of ILK, PAX, BRAF, ERK and PKC. Indeed, the results showed suggest that the HLA-DR mediated signalling provides a platform useful to frustrate an effective anti-tumour response and to increase melanoma migration and metastatic dissemination of this cancer.



**Keywords:** MHC class II; melanoma; lipid raft; PD-L1; signalling pathways; migration.

**Running title:** HLA-DR signalling in melanoma cells.

## 1. Introduction

Melanoma, is one of the most aggressive cancers worldwide, its incidence rates increase rapidly in western population and unfortunately it is responsible of about 20% overall cancer mortality as well as of approximately 80% skin cancer related mortality [1]. Indeed, the chemotherapeutic treatment has a generally low effect on survival rates of melanoma patients, but recently targeted therapies and immunotherapies, changed significantly the therapeutic choices of metastatic melanoma patients. In particular, the discovery and the U.S. Food and Drug Administration (FDA) approval of immune checkpoint target antibodies such as the monoclonal antibodies directed against the cytotoxic T Lymphocyte Antigen 4 (CTLA-4) (ipilimumab) [2], the Programmed Cell Death 1 (PD-1) (nivolumab and pembrolizumab) [3, 4] and a member of the B7 family, the Programmed Cell Death Ligand 1 (PD-L1, B7-H1) (atezolizumab) [5] receptors, inducing responses in 20–40% of patients, changed significantly the landscape of metastatic melanoma treatments. Indeed, the PD-1/PD-L1 interaction during antigen presentation of APC and cancer, inhibit the expansion and survival of antitumor T cells as well as the T-cell cytotoxic activity ultimately facilitating immune evasion [6]. Instead, the antibodies that prevent the binding of T-cell receptor PD-1 with the highly melanoma expressed PD-L1 lead to the reactivation of T-cells anti-tumour function in tumour microenvironment [3, 4]. Nevertheless, the T cells reactivation could be ineffective in a tumour microenvironment heterogeneous as well as in immune resistant cancers. PD-L1 as well as the Major Histocompatibility Complex (MHC) class II molecules is expressed constitutively and after interferon-γ (IFNγ) stimulation in a wide number of cells including some cancer cells [7, 8]. In particular, the MHC class II molecules are expressed in almost 50% of melanoma and although the MHC class II molecules expressed in melanoma cells may directly present tumour antigens to CD4+ T cells and trigger their effector functions [9], the constitutive MHC class II expression in melanoma is related to a bad prognosis [10-13]. The MHC class II molecules are signalling receptors whose engagement leads to the activation of several signalling pathways by tyrosine and serine/threonine kinases phosphorylation such as Src, Syk,

Extracellular signal-Regulated Kinase (ERK) and Protein Kinase C (PKC) [14, 15]. Furthermore, the class II signalling is transduced through Inositol lipid hydrolysis, production of cyclic AMP (cAMP) or mobilisation of intracellular Ca2+, ultimately leading to APCs activation or death [16-19]. Interestingly, the synergy between the class II molecules and several transmembrane receptors and signalling proteins is enabled by the lipid raft localisation of these molecules [20]. The lipid rafts are specific microdomains of the plasma membrane enriched in freely diffusing, stable assemblies of sphingolipids (sphingomyelin and glycosphingolipids) and cholesterol implicated in selective protein–protein interactions as well as in the assembly of transient signalling platforms [21]. Notably, in cancer cells, the lipid rafts localisation and activation of a wide number of receptors such as Integrins and Cell Adhesion Molecules (CAMs) as well as of some signalling molecules is related, for example, to the regulation of cell adhesion and migration during each phase of cancer development and progression [21-23]. In particular, the aggressive metastatic trend of melanoma is associated to the lipid raft recruitment of these molecules but also to their improved expression, to the deregulation of their functions and to the adhesion receptors-mediated interaction between tumour cells and cellular blood components, that promotes the tumour cells detachment from primary tumour and facilitate the survival of melanoma cells in the vascular system. Indeed, Integrins α/β are heterodimeric transmembrane receptors that mediating the cells anchorage to extracellular matrix (ECM), interacting with cytoskeleton and through the activation of downstream signalling pathways, play a key role in cell adhesion, migration, invasion, cell survival, growth and gene expression [24]. In particular, the Integrin functions are mediated by the interaction with structural proteins such as Paxillin (PAX), Talin and Vinculin and modulate the regulation of signalling pathways such as Focal Adhesion Kinase (FAK), Integrin-Linked Kinase, Src kinases, the ERK/Mitogen-Activated Protein Kinase (MAPK) and the Phosphoinositide 3-kinase/AKT pathways [24]. In human metastatic melanoma the α2, α5, αV, β1 and β3 Integrin subunits are up regulated and between them the Integrin β3 is one of the most specific markers of vertical growth phase of melanoma [25]. Furthermore, Melanoma Cell Adhesion Molecule (MCAM, MUC18 or

CD146) a marker of melanoma metastatic progression and Intercellular Adhesion Molecule-1 (ICAM-1) are transmembrane receptors often over expressed in advanced primary and metastatic human melanoma cells [26]. In particular, MCAM mediates homotypic melanoma adhesion and, as well as ICAM, the heterotypic adhesion between melanoma, leukocytes and endothelial cells, promoting the formation of cellular clumps and allowing melanoma to survive in lymphatic and vascular systems. Furthermore, MCAM and ICAM adhesion receptors mediate the interaction of melanoma cells with vessel walls thus supporting the melanoma cells intravasation and extravasation in distant organs [24, 27]. In this contest, the aim of our work was to understand the consequences on melanoma metastatic progression, migration and immune escape of HLA-DR mediated signalling. Notably, we previously reported the increased lipid rafts recruitment of HLA-DRα in melanoma cells after HLA-DR engagement [28] and we and others reported that the signalling activated by MHC class II molecules is associated in different tumour cell lines, to the lipid rafts localisation of these molecules [20, 28]. Furthermore, the HLA-DR mediated signalling could inhibit, in melanoma cells, the Fas mediated apoptosis [14]. Therefore, in this paper we reported, in response to a sustained continuous HLA-DR stimulation, the increased expression of HLA-DRα, MCAM, ICAM, Integrin β1, Integrin β3, Integrin α5, Integrin α□ and PD-L1 receptors. We also showed the increased expression of FAK, PAX, BRAF, ERK, ILK, AKT, PKC and Signal Transducers and Activators of Transcription 3 (STAT3) signalling proteins, the increased activation of FAK, PAX, ERK, AKT, PKC and STAT3 as well as the lipid rafts recruitment of HLA-DRα, MCAM, ICAM, Integrin β1, Integrin β3, PD-L1, FAK, AKT and STAT3 in response to a sustained continuous HLA-DR stimulation. Furthermore, we identified the HLA-DR mediated signals depending on lipid rafts integrity through the treatment of melanoma cells with the methyl-β-cyclodextrin (MβCD) that disrupt the lipid raft domains through cholesterol depletion. Finally, we reported that the HLA-DR mediated signalling increases the melanoma cell migration and invasion, thus suggesting a new role played by the HLA-DR molecules on metastatic dissemination and on immune evasion of melanoma.

## 2. Material and Methods

### 2.1. Cell lines, antibodies and reagents

A375 (ATCC-CRL-1619) [29], HT-144 (ATCC-HTB-63), M74 melanoma cell lines (kindly given by Prof. C. Alcaide-Loridan, Paris Diderot University, Paris, Institut Jacques Monod) and LAN-5 neuroblastoma cells (kindly given by Dr. M. Di Carlo, IBIM-CNR) were grown in RPMI 1640 supplemented with 10% FCS and 1% penicillin-streptomycin (10,000 U/ml and 10,000 μg/ml, respectively) in 5% $CO_2$ at 37°C. The mouse monoclonal antibody direct against the C-terminal intracellular tail of the HLA-DR-α-chain (DA6.147) [30] was a kind gift of Prof. C. Alcaide-Loridan. Rabbit polyclonal antibody direct against caveolin-1 (clone pAb) and mouse monoclonal antibodies direct against the HLA-DR (clone G46-6, mouse IgG2a), pTyr-397 FAK motif, Integrin β1, Integrin α5, Integrin αV, ICAM-1, MCAM, and PAX were obtained from BD Biosciences (Lexington, KY). The rabbit polyclonal antibodies direct against Integrin β3, FAK, MCAM, STAT3, Pdcd-1L1 and PKCα as well as the mouse monoclonal antibody direct against Raf-B, ILK and pSTAT3 (Tyr-705) were purchased from Santa Cruz (Santa Cruz, CA, USA). The rabbit polyclonal antibodies direct against Phospho-PKC pan (γThr514), Phospho-PAX (Tyr-118), AKT, pAKT (Ser-473), p44/42 MAPK and Phospho-p44/42 MAPK (Thr-202/Tyr-204) were obtained from Cell Signaling Technology. Anti-β-actin mouse monoclonal antibody and the mouse IgG2a isotype control Ig were obtained from Sigma (St Louis, MO). Infrared dye-conjugated IRDye800 anti-mouse and anti rabbit (LI-COR Biosciences) as well as anti-mouse and anti-rabbit Alexa Fluor 680 conjugated (Molecular Probes) were purchased as secondary antibodies. All other chemicals were of analytical grade and were purchased from Sigma Chemical Co., Merck/VWR, or J. T. Baker (Phillipsburg, NJ).

### 2.2. Preparation of total cell extracts and isolation of raft fractions

Semi-confluent cells were stimulated with 1 μg/ml of the mouse L243 antibody direct against a conformational epitope of the HLA-DR molecules [31] for 24h, 48h and 72h or with 1 μg/ml of isotype-matched control antibody for 48h. To obtain total cell extracts, the cells were lysed on ice for 30 min in RIPA buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% NP40, 0,5% DOC, 0,1% SDS) containing proteases inhibitors (4 mM PMSF and protease inhibitors cocktail, Sigma) and phosphatase inhibitors (cocktail 2 and 3 of phosphatase inhibitors, Sigma) and then cleared of cellular debris by centrifugation at 12,000x g at 4°C for 30 min. The protein concentration of the supernatants was determined using the Bradford protein assay (Bio-Rad laboratories GmbH, München, Germany). Otherwise, to isolate the raft fractions [28, 32], the cells were lysed in MBS buffer (25 mM MES, 2 mM EDTA pH8 and 150 mM NaCl) containing 1% Triton X100, protease and phosphatase inhibitors for 30 min on ice. The lysates mixed with an equal volume of 85% sucrose (w/v) in MBS buffer, were placed at the bottom of a polycarbonate ultracentrifuge tube (Beckman Instruments, Palo Alto, CA), overlaid with 2 ml of 35% sucrose and 1 ml of 5% sucrose in MBS buffer containing 2 mM EDTA pH 8, protease and phosphatase inhibitors and were centrifuged at 100,000x g for 20h at 4°C in a SW55Ti rotor (Beckman Instruments, Palo Alto, CA). Nine fractions of 550 μl/each were collected from the top of the discontinuous sucrose gradient.

### 2.3. Western blot analysis

40 μg of total cell extracts or 28 μl of raft fractions were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and absorbed to nitrocellulose membrane (Hybond ECL, GE Healthcare, Biosciences). Blocking was performed in blocking buffer (LI-COR Biosciences) overnight at 4°C in TBS buffer. Incubation with primary antibodies diluted in 1:1 blocking solution (LI-COR Biosciences) and TBS-Tween buffer, was at room temperature for 1h 30 min. The membranes were incubated with infrared dye-conjugated IRDye800 (LI-COR Biosciences) or with Alexa Fluor 680 conjugated (Molecular Probes) anti-mouse and anti-rabbit infrared fluorescent-labelled secondary antibodies at room temperature for 1h 30 min. The

membranes were processed and protein bands were scanned and quantified using the Odyssey infrared imaging system (LI-COR Biosciences).

### 2.4. Cell migration and invasion assays

$2x10^4$ or $2.5x10^4$ melanoma cells stimulated with 1 μg/ml of mouse L243 antibody [31] for 32h or 26h, were plated in serum-free medium with or without L243 antibody, in the inner chamber of a 24-well culture plate (Falcon) with a polyethylene terephthalate (PET) membrane (pore size, 8μm; Falcon) respectively uncoated (migration assay) or coated with matrigel (invasion assay) (Falcon) [33]. The lower wells were filled with RPMI supplemented with 10% FBS and cell migration across the porous filter or cell invasion across matrigel layer was allowed for respectively 16h or 22h at 37°C (tot 48h of stimulation). The cells in the upper chamber were removed with a cotton swab and the migrated cells attached to the lower surface of the transwell membrane were fixed with 100% methanol for 20 min and stained with 0.5% crystal violet for 1h. After staining, all the cells on the lower side of the filters were counted under phase contrast microscope (Leica).

### 2.5. Wound healing assay

Confluent A375 and HT-144 melanoma cells were scratch-wounded using a 200μl pipette tip [33]; the cells were washed twice with PBS and incubated with or without 1 μg/ml of L243 antibody in RPMI supplemented with 10% FBS, for 24h and 48h at 37°C. The plates were visualised by inverted phase contrast microscopy (Leica) at 0h, 24h and 48h, respectively T0, T1 and T2. The images of wound closure were captured using digital imaging and the area free of cells was assessed by digitally drawn lines at the wound edges.

## 3. Results

**3.1. HLA-DR mediated signalling increases the expression of HLA-DRα, Integrin and CAM receptors.**

The signalling mediated by the HLA-DR molecules was studied in A375 and HT-144 human melanoma cell lines, constitutively expressing the MHC class II molecules. The A375 and HT-144 cell lines were stimulated for 24h, 48h and 72h with 1 μg/ml of an anti-class II antibody (L243) that recognise a conformational epitope of the HLA-DR molecules [31]. Therefore, in L243 stimulated and unstimulated cells (NS) we analysed the expression of HLA-DRα molecules (Fig. 1A) as well as of MCAM, ICAM (Fig. 1B and 1C), Integrin β1, β3, α5 and αV (Fig. 2A and 2B) adhesion receptors. The results of the western blot experiments showed in both melanoma cell lines, an increased expression of HLA-DRα molecules (Fig. 1A) with a pick ranging from 24h to 48h after L243 stimulation compared to unstimulated cells (Fig. 1A, NS). Interestingly we also showed in both melanoma cell lines, an increased expression of MCAM and ICAM (Fig. 1B and 1C) as well as of Integrin β1, β3, α5 and αV (Fig. 2A and 2B) with a pick ranging from 48h to 72h after L243 stimulation compared to unstimulated cells (Fig. 2A and 2B, NS). Furthermore, in A375 and HT-144 cell lines stimulated with an isotype control antibody (IgG2a) or left unstimulated (NS) we showed, as a control of the specificity of the class II mediated signalling, that the expression of Integrin β1, Integrin β3, MCAM and ICAM adhesion receptors, is comparable in isotype control stimulated and unstimulated cells (Suppl. Fig. 1 and 3) but increases in A375 and HT-144 cells stimulated in parallel with the L243 antibody (Suppl. Fig. 1 and 3). The expression of adhesion receptors was also analysed in M74 [12] melanoma (Integrin β1, Integrin β3, MCAM and ICAM) and LAN-5 [34] neuroblastoma (Integrin β1 and Integrin β3) MHC class II negative cell lines stimulated with the L243 (L243) antibody. The results obtained showed that the expression of adhesion receptors analysed is comparable in MHC class II negative cell lines stimulated with the L243, the isotype control antibodies or left unstimulated (Suppl. Fig. 5). Furthermore to ensure that equal amounts of cell extracts were used in each sample, western blot experiments were performed

using an antibody specific for the β-actin protein (Fig. 1A, 1B, 2A, 2B, Suppl. Fig. 1, 3 and 5 lower panels). The media of the values obtained after proteins quantification normalized for actin content of different batches of cell extracts were presented as a single graphic for each receptor (Fig 1A, 1B, 2A and 2B). These results showed a tight and coordinated regulation of the expression of HLA-DRα, Integrin and CAM adhesion receptors exerted by the HLA-DR mediated signalling as well as the specificity of the anti HLA-DR mediated signalling, thus strongly suggesting that the class II mediated signalling could promote melanoma metastatic dissemination.

**3.2. Signalling mediated by the HLA-DR molecules.**

The Integrin signalling leads to the activation of ILK/FAK/PAX, PI3K/AKT and BRAF/MEK/ERK signalling pathways and in particular, induces the activation of FAK through the auto-phosphorylation of Tyr-397 and of PAX through the FAK mediated phosphorylation of Tyr-118 [35]. Therefore, in melanoma cells unstimulated and stimulated for 24h, 48h and 72h, we studied the expression of ILK, FAK and PAX, the tyrosine phosphorylation of FAK and PAX and, as downstream effectors of these signalling proteins, the expression of BRAF and the expression and activation of ERK1/2 and AKT proteins (Fig. 3A, 3B and 4A). Interestingly, the results of the western blot experiments showed, in both cell lines, a growing expression of ILK, PAX and FAK (Fig. 3A) and also an increasing phosphorylation of FAK and PAX respectively on Tyr-397 and on Tyr-118 as a consequence of the HLA-DR engagement (Fig. 3A). Moreover, the results obtained showed in both cell lines a growing expression of BRAF, ERK1/2 and AKT (Fig. 3B and 4A) and also an increasing phosphorylation of ERK1/2 on Thr-202/Tyr-204 and AKT on Ser-473, the hallmark of AKT activation [36], in response to HLA-DR stimulation (Fig. 3B and 4A). Interestingly, the results obtained showed in A375 cells a largest increase of ILK expression and of FAK and PAX expression and activation compared to HT-144 cells, while in HT-144 cells, our results showed a largest increase of AKT expression and activation compared to A375 cells (Fig. 3 and 4A) as a consequence of HLA-DR engagement. Indeed, the HLA-DRβ chain contains some

motifs necessary and sufficient for the activation in B cells of PKC protein [17], a key component of signalling pathways regulating proliferation, survival and apoptosis on several cell types [37]. Therefore, the involvement of PKC in MHC class II mediated signalling pathway was also examined in melanoma cells. The results of the western blot experiments that we performed showed the increased expression of the PKC alpha isoform and the increased activation of conventional and novel PKC isoforms in both A375 and HT-144 melanoma cells stimulated for 24h, 48h and 72h with the L243 antibody (Fig. 4B). Interestingly, the melanoma cells expressing the HLA-DR molecules are often PD-L1 positive also in absence of IFNγ stimulation [38]. Moreover, STAT3, MAPK and PI3K/AKT signalling pathways activate the expression of PD-L1 receptor [39, 40], therefore, we analysed in A375 and HT-144 melanoma cells stimulated for 24h, 48h and 72h with the L243 antibody or left unstimulated (NS) the expression of STAT3 and PD-L1 (Fig. 4C and 4D). The results obtained, showed an increased expression of STAT3 and PD-L1 (Fig. 4C and 4D) and the activation of STAT3 (Fig, 4C, pSTAT3) after 48h and 72h of HLA-DR stimulation respectively in A375 and HT-144 cells, strongly suggesting a correlation between the STAT3 activation and PD-L1 expression in response to HLA-DR mediated signalling. Furthermore, in A375 and HT-144 cell lines stimulated with an isotype control antibody (IgG2a) or left unstimulated (NS) we showed, as a control, that the expression of STAT3, AKT, HLA-DRα and PD-L1 molecules, is comparable in isotype control stimulated and unstimulated cells (Suppl. Fig. 2 and 4) but increases in A375 and HT-144 cells stimulated in parallel with the L243 antibody (Suppl. Fig. 2 and 4). The expression of STAT3 and AKT molecules was analysed, as a control, in M74 [12] melanoma and LAN-5 [34] neuroblastoma MHC class II negative cell lines stimulated with the L243 (L243) antibody. The results obtained showed that the expression of the signalling molecules analysed is comparable in MHC class II negative cell lines stimulated with the L243, the isotype control antibodies or left unstimulated (Suppl. Fig. 6), thus demonstrating the specificity of the signalling activated by the anti HLA-DR antibody in class II positive cells. Finally, western blot experiments using an antibody specific for the β-actin protein were performed to ensure that equal amounts of cell

extracts were used in each sample (Fig. 3A, 3B, 4A, 4B, 4C, 4D, Suppl. Fig. 2, 4 and 6 lower panels). In conclusion, these results collectively indicate that the HLA-DR mediated signalling could directly or indirectly activate ILK/AKT, FAK/PAX/AKT, BRAF/ERK, PKC and STAT3/PD-L1 signalling pathways promoting not only melanoma cells proliferation and migration but also melanoma immune escape.

**3.3. The lipid raft compartments of HLA-DR stimulated A375 and HT-144 melanoma cells.**

The receptors and signalling molecules studied are often localized in membrane microdomains, the lipid raft compartments, implicated in selective protein–protein interactions and in the assembly of transient signalling platforms [21]. Therefore, in A375 and HT-144 melanoma cells we analysed the membrane localisation of receptors and signalling proteins in response to HLA-DR signalling. The MHC class II constitutive expressing A375 and HT-144 melanoma cell lines were stimulated for 24h and 48h with the L243 antibody or left unstimulated (Fig. 5 and 6) and the lipid raft compartments were isolated through discontinuous sucrose gradient [28, 32]. The lipid raft compartments localised on fractions F2, F3 and F4 of the sucrose gradient, were analysed by western blot experiments. The results obtained, showed that only a slight amount of HLA-DRα molecules was constitutively localised in the low density fractions (F2, F3 and F4, Fig. 5A and 6A, NS, higher panels) containing the lipid raft compartments, while a significant recruitment of the HLA-DRα molecules in these fractions (F2, F3 and F4, Fig. 5A and 6A, L243, higher panels) was observed after L243 stimulation of A375 (Fig. 5A) and HT-144 (Fig. 6A) melanoma cells for 24h (Fig. 5A and 6A, left panels) and 48h (Fig. 5A and 6A, right panels). Furthermore, in A375 melanoma cells stimulated with L243 for 24h (left panels), we observed the lipid raft increased localisation of Integrin β1 and β3 as well as of FAK, STAT3 and also PD-L1 (Fig. 5A and 5B, L243), compared to unstimulated cells (Fig. 5A and 5B, NS). Moreover, we showed the increased lipid raft localisation in A375 melanoma cells stimulated for 24h (left panels) and to a lesser extent for 48h (right panels), of MCAM and AKT (Fig. 5B, L243) compared to unstimulated cells (Fig.

5A and 5B, NS). In HT-144 melanoma cells, the western blot experiments showed the increased lipid raft localisation of ICAM, Integrin β3 and FAK after 24h (Fig. 6A, L243) and also 48h of stimulation (Fig. 6A, L243), compared to unstimulated cells (Fig. 6A, NS). The STAT3, AKT and PD-L1 lipid rafts localisation was increased in HT-144 melanoma cells after 24h of L243 stimulation (Fig. 6B, L243), compared to unstimulated cells (Fig. 6B, NS). As a positive control of the raft compartments the amount of the caveolin localised on each isolated fraction [32] was also analysed (Fig. 5A 5B, 6A and 6B lower panels). In conclusion, these results showed that the HLA-DR mediated signalling increase the recruitment on the lipid raft compartments of the HLA-DR molecules, Integrin β1, Integrin β3, MCAM and ICAM adhesion receptors, FAK, STAT3 and AKT signalling molecules as well as the PD-L1 receptor in melanoma cells.

**3.4. Effects of MβCD induced lipid rafts destruction on HLA-DR mediated signalling.**

In the aim to verify if the HLA-DR mediated signalling pathways arise from the HLA-DR molecules localised in the lipid raft fractions, we treated the A375 and HT-144 cell lines with the methyl-β-cyclodextrin (MβCD) that disrupts the lipid raft integrity through cholesterol depletion. In both melanoma cell lines stimulated with the L243 antibody and treated with 1 mM MβCD for 24h and 48h we analysed the expression of HLA-DRα, Integrin β1, Integrin β3 and PD-L1 receptors as well as of FAK and STAT3 signalling molecules, while the expression of MCAM and ICAM adhesion receptors was analysed respectively on A375 (Fig. 7B) and HT-144 melanoma cells (Fig. 8B). The results of the western blot experiments showed that in A375 the HLA-DRα expression, was at steady state level after 24h and decreased after 48h of L243 stimulation and MβCD treatment (Fig. 7A, L243 + MβCD). In HT-144 cells the expression of HLA-DRα molecules was at steady state level (Fig. 8A, L243 + MβCD) after 24h and 48h of L243 stimulation and MβCD treatment compared to control cells (Fig. 7A and 8A, NS). Otherwise, the expression of FAK, STAT3 and PD-L1 as well as the phosphorylated form of FAK decreased in both melanoma cell lines to a

greater extent after 48h than after 24h of L243 stimulation and MβCD treatment (Fig. 7C and 8C, L243 + MβCD). In A375 cells the expression of MCAM as well as of Integrin β1 (Fig. 7B) and in HT-144 cells the expression of ICAM (Fig. 8B) decreased after 48h of L243 stimulation and MβCD treatment with a temporarily increase after 24h of treatment and stimulation (Fig. 7B and 8B, L243 + MβCD). Instead, the Integrin β1 and Integrin β3 expression increased after 24h and 48h of stimulation and treatment of HT-144 cells (Fig. 8B, L243 + MβCD) and the Integrin β3 expression, increased in A375 only after 48h of stimulation and treatment (Fig. 7B, L243 + MβCD) suggesting a different pattern of regulation for these proteins. Finally we performed western blot experiments using an antibody specific for the β-actin protein, in the aim to ensure that equal amounts of cell extracts were used in each sample (Fig. 7A, 7B, 7C, 8A, 8B and 8C, lower panels). These data strongly suggested that the expression of Integrin β1 in A375, ICAM in HT-144 and MCAM, HLA-DRα, PD-L1, STAT3, FAK as well as the activation of FAK in both cell lines, requires the integrity of lipid raft compartments and therefore, that their expression is mediated by the signalling activated by the HLA-DR molecules localised in the lipid raft compartments. Otherwise, the results reported strongly suggested that the expression of Integrin β1 (HT-144) and Integrin β3 (A375 and HT-144) is mediated by the signalling activated by the HLA-DR molecules localised outside the lipid raft compartments.

### 3.5. The HLA-DR signalling increases the migration and invasion of melanoma cells

Cell migration is a multistep process managed by a complex network of signal transduction pathways involving adhesion receptors and signalling molecules, that play a key role on the aggressive metastatic trend of melanoma [24]. Therefore, in the aim to verify the role of HLA-DR mediated signalling in the metastatic dissemination of melanoma cells we analysed the migration and invasion of stimulated melanoma cells compared to unstimulated cells. The results of migration assay showed that the migration of A375 (Fig. 9A, middle panel) and HT-144 cells (Fig. 9A, right

panel) increased significantly after 48h of L243 (L243) stimulation (71% and 69%, respectively), compared to unstimulated cells (NS, Fig. 9A middle and right panels). The migration of A375 and HT-144 melanoma cells was analysed also through *in vitro* scratch wound repair assay (Fig. 9B) and the results obtained showed that the A375 and HT-144 cells stimulated (L243) for 48h (T2), invaded the denuded area approximately twofold than unstimulated cells in the same time (NS, Fig. 9B, higher and middle panels). Likewise, a large number (88%) of A375 melanoma cells stimulated for 48h (L243) invaded the matrix layer compared to unstimulated cells (NS) (Fig. 9A, right panel). In conclusion, our results show that the signalling activated by the HLA-DR molecules increases the migration and invasion of melanoma cells thus playing a main role on the regulation of metastatic dissemination of melanoma.

## 4. Discussion

The constitutive MHC class II expression is restricted to professional APCs such as dendritic cells, B lymphocytes, macrophages and thymic epithelial cells, nevertheless, almost 50% of melanomas express constitutively the MHC class II molecules. Indeed, human cancer cells often use the loss of MHC class II expression to escape the control of immune cells, instead in melanoma, nevertheless the MHC class II-peptide complex trigger the effector functions of CD4+ T cells, tumour-reactive T cells are associated to in vivo tumour regression in only a modest number of patients [9, 41]. Melanoma is a cancer highly immunogenic that can evade host immune surveillance inhibiting an effective immune response by the active suppression of tumour-reactive T-cell clones [41]. Therefore, in the light of the hypothesis that the MHC class II constitutive expression in melanoma cells could be associated to immune escape and metastatic progression of melanoma cells, we studied the HLA-DR mediated signalling activated by a specific antibody that mimics the interaction of TCR with class II molecules [31]. In particular we showed that the signalling mediated by the HLA-DR molecules through a sustained continuous class II stimulation of human melanoma cells, is associates to a significant increase of HLA-DR$\alpha$, Integrin and CAM

adhesion receptors expression and also to their lipid rafts recruitment. Accordingly to the reported data showing that the class II crosslinking mediates the aggregation between homologous APCs and also between melanoma cells [42, 43], we could speculate that the HLA-DR mediated increased expression of Integrin and CAM receptors could be involved in homologous and heterologous interactions between melanoma and endothelial cells but also between melanoma and T cells. Furthermore, the MHC class II mediated increased expression of adhesion receptors could allow the melanoma cells to assemble in clumps that could survive in lymphatic and vascular systems, thus supporting the melanoma cells intravasation, extravasation and melanoma metastatic spread. Therefore, we propose that the class II engagement provides foci of signalling in melanoma cells through the lipid rafts recruitment and activation of HLA-DR and adhesion receptors. Interestingly, the clustering of Integrins is associated to the activation of the tyrosine kinase FAK as well as of the serine/threonine kinase and adaptor/scaffold protein ILK, associated temporally and spatially with the cytoplasmic tail of Integrin β [44, 45]. In particular, the phosphorylated form of FAK recruits and activates the signal transduction protein PAX, thus leading to the activation of the downstream effectors BRAF/ERK and AKT [35]. In the light of the data reported, we analysed the HLA-DR mediated signalling and we showed the activation of ILK/AKT, FAK/PAX/AKT and BRAF/ERK signalling pathways as well as the lipid rafts recruitment of FAK and AKT proteins in response of the HLA-DR mediated signalling. Furthermore, we showed that the HLA-DR mediated increased expression of Integrin β1 in A375, ICAM in HT-144 and MCAM, HLA-DRα, PD-L1, STAT3, FAK as well as the activation of FAK in both cell lines, is dependent on melanoma lipid raft integrity. Therefore, these results show that the HLA-DR molecules modulate their functions through signalling platforms such as the lipid raft compartments. Indeed, the interaction of melanoma cells with the microenvironment is thought to be crucial for their metastatic dissemination and our results suggest that the class II mediated signalling enhancing the expression of Integrin and CAM adhesion receptors, activating the signalling associated and increasing the motility and invasion of melanoma cells, modulates multiple cell invasion-associated intracellular

processes based also on increased integrin functions. Furthermore, we showed the HLA-DR mediated increased expression of PKCα and□□□□ activation of the conventional and novel PKC isoforms. Notably, the activation of the PKC signalling pathways enable the phosphorylation and activation of STAT3 but also the ERK mediated activation of PI3K/AKT signalling [46, 47]. Therefore, our results suggest the involvement of PKC proteins in class II mediated activation of MAPK and AKT signalling as well as in the increased expression of STAT3 transcription factor. Interestingly, the signalling mediated by STAT3 induces the expression of genes implicated in cells survival, proliferation, invasion and metastasis [48] as well as of cytokines, chemokines and growth factors that interfere with the function of innate and adaptive immunity, thus promoting the cancer cells immune escape [49]. Indeed, STAT3 inhibits the MHC class II expression in murine bone-marrow-derived DCs [50] and in primary human blood macrophages [51], but STAT3 cannot inhibit the class II expression in WM115 and in WM98-1 melanoma cells [9, 52, 53] or, as we reported in this paper, in A375 and HT-144 cell lines. Instead, in melanoma cells the HLA-DR mediated signalling increases the expression, the activation as well as the lipid rafts localisation of STAT3 suggesting a class II mediated STAT3 signalling that could strongly modify the tumour microenvironment and the innate and adaptive immunity. In addition, the STAT3 transcription factor as well as the MAPK and AKT signalling pathways, regulate the expression of PD-L1 receptor [39, 40]. Indeed, PD-L1 receptor activates the T-cell inhibitory pathways thus inhibiting the T-cell cytotoxic activity and facilitating cancer immune evasion [6]. Notably, in both melanoma cell lines we showed the HLA-DR mediated increased expression as well as the lipid raft recruitment of PD-L1 receptor. Therefore, these results suggest a novel mechanism of PD-L1 regulation exerted by class II molecules in melanoma cells that, through the STAT3 mediated activation of PD-L1 receptor expression, could allow the melanoma immune escape. Finally, we could speculate that in the aim to recruit tumour-specific CD4+ T cells and stabilize the melanoma-T cells interaction, the HLA-DR mediated signalling increase the expression and the lipid raft recruitment of HLA-DR, PD-L1 and adhesion receptors, thus increasing the strength, duration and

effectiveness of this interaction. The consequence of melanoma-T cell interactions could be the activation in T cells of inhibitory signals mediated by PD-L1 and in melanoma cells the activation of some signalling pathways that deeply modify the tumour microenvironment supporting the class II mediated metastatic progression. In conclusion, our results suggest that the MHC class II mediated signalling could interfere with the effectiveness of the checkpoint immunotherapy, making ineffective the T cells reactivation strategies, increasing the immune evasion and the metastatic dissemination of melanoma.

## Conflict of Interest

The authors declare no conflict of interest.

## Author contributions

FC designed, acquired and analysed data, performed experiments and contributed to the draft of manuscript; GB designed, acquired and analysed data, performed experiments and drafted the manuscript. The authors read and approved the final manuscript.

## Grant Support

This work was supported by the “Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR) (MERIT/FIRB Project RBNE08PA9T).

## Acknowledgements

We wish to thank Prof C. Alcaide-Loridan (Paris Diderot University, Paris, Institut Jacques Monod) for providing melanoma cell lines and anti-HLA-DR antibodies, Dr. M. Di Carlo (IBIM-CNR) for

providing LAN-5 neuroblastoma cell line, Dr. F. Di Leo and Mr. L.Caruana (IBIM-CNR) for technical support.

## References

[1] Miller KD, Siegel RL, Lin CC, Mariotto AB, Kramer JL, Rowland JH, Stein KD, Alteri R, Jemal A, Ca-Cancer J Clin. 2016;66:271-289.
[2] Schadendorf D, Hodi FS, Robert C, Weber JS, Margolin K, Hamid O, Patt D, Chen TT, Berman DM, Wolchok JD, J Clin Oncol. 2015;33:1889-1894.
[3] Robert C, Ribas A, Wolchok JD, Hodi FS, Hamid O, Kefford R, Weber JS, Joshua AM, Hwu WJ, Gangadhar TC, Patnaik A, Dronca R, Zarour H, Joseph RW, Boasberg P, Chmielowski B, Mateus C, Postow MA, Gergich K, Elassaiss-Schaap J, Li XN, Iannone R, Ebbinghaus SW, Kang SP, Daud A, Lancet. 2014;384:1109-1117.
[4] Topalian SL, Sznol M, McDermott DF, Kluger HM, Carvajal RD, Sharfman WH, Brahmer JR, Lawrence DP, Atkins MB, Powderly JD, Leming PD, Lipson EJ, Puzanov I, Smith DC, Taube JM, Wigginton JM, Kollia GD, Gupta A, Pardoll DM, Sosman JA, Hodi FS, J Clin Oncol. 2014;32:1020-1030.
[5] Lu J, Lee-Gabel L, Nadeau M, Ferencz T, Soefje S, J. Oncol. Pharm. Pract. 2015;21:451–467.
[6] Thompson RH, Gillettt MD, Cheville JC, Lohse CM, Dong HD, Webster WS, Krejci KG, Lobo JR, Sengupta S, Chen LP, Zincke H, Blute ML, Strome SE, Leibovich BC, Kwon ED, P Natl Acad Sci USA. 2004;101:17174-17179.
[7] Tsujisaki M, Igarashi M, Sakaguchi K, Eisinger M, Herlyn M, Ferrone S, J. Immunol. . 1987;138:1310-1316.
[8] Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, Roche PC, Lu J, Zhu G, Tamada K, Lennon VA, Celis E, Chen L, Nature medicine. 2002;8:793-800.
[9] Donia M, Andersen R, Kjeldsen JW, Fagone P, Munir S, Nicoletti F, Andersen MH, Straten PT, Svane IM, Cancer research. 2015;75:3747-3759.
[10] Elder DE, Rodeck U, Thurin J, Cardillo F, Clark WH, Stewart R, Herlyn M, Cancer research. 1989;49:5091-5096.
[11] Bernsen MR, Hakansson L, Gustafsson B, Krysander L, Rettrup B, Ruiter D, Hakansson A, British journal of cancer. 2003;88:424-431.
[12] Deffrennes V, Vedrenne J, Stolzenberg MC, Piskurich J, Barbieri G, Ting JP, Charron D, Alcaide-Loridan C, Journal of immunology. 2001;167:98-106.
[13] Mendez R, Aptsiauri N, Del Campo A, Maleno I, Cabrera T, Ruiz-Cabello F, Garrido F, Garcia-Lora A, Cancer Immunol Immun. 2009;58:1507-1515.
[14] Aoudjit F, Guo W, Gagnon-Houde JV, Castaigne JG, Alcaide-Loridan C, Charron D, Al-Daccak R, Experimental cell research. 2004;299:79-90.
[15] Mourad W, Geha RS, Chatila T, The Journal of experimental medicine. 1990;172:1513-1516.
[16] Guo WY, Castaigne JG, Mooney N, Charron D, Al-Daccak R, Eur J Immunol. 2003;33:928-938.
[17] Harton JA, Van Hagen AE, Bishop GA, Immunity. 1995;3:349-358.
[18] Lane PJL, Mcconnell FM, Schieven GL, Clark EA, Ledbetter JA, Journal of immunology. 1990;144:3684-3692.
[19] Truneh A, Albert F, Golstein P, Schmitt-Verhulst AM, Nature. 1985;313:318–320.
[20] Huby RD, Dearman RJ, Kimber I, The Journal of biological chemistry. 1999;274:22591-22596.
[21] Mollinedo F, Gajate C, Adv Biol Regul. . 2015;57:130-146.
[22] Hashimoto N, Hamamura K, Kotani N, Furukawa K, Kaneko K, Honke K, Furukawa K, Proteomics. 2012;12:3154-3163.
[23] Wang RF, Bi JJ, Ampah KK, Zhang CM, Li ZY, Jiao Y, Wang XR, Ba XQ, Zeng XL, Int J Biochem Cell B. 2013;45:1679-1689.
[24] Alizadeh AM, Shiri S, Farsinejad S, Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine. 2014;35:8483-8523.
[25] Natali PG, Nicotra MR, Bartolazzi A, Cavaliere R, Bigotti A, Int J Cancer. 1993;54:68-72.
[26] Johnson JP, Stade BG, Holzmann B, Schwäble W, Riethmüller G, Proc Natl Acad Sci U S A. 1989;86:641-644.
[27] Xie SH, Luca M, Huang SY, Gutman M, Reich R, Johnson JP, BarEli M, Cancer research. 1997;57:2295-2303.
[28] Barbieri G, Rimini E, Costa MA, International journal of oncology. 2011;38:1589-1595.
[29] Giard DJ, Aaronson SA, Todaro GJ, Arnstein P, Kersey JH, Dosik H, Parks WP, Journal of the National Cancer Institute. 1973;51:1417-1423.
[30] Guy K, Van Heyningen V, Cohen BB, Deane DL, Steel CM, Eur J Immunol. 1982;12:942-948.
[31] Lampson LA, Levy R, Journal of immunology. 1980;125:293-299.
[32] Xavier R, Brennan T, Li Q, McCormack C, Seed B, Immunity. 1998;8:723-732.
[33] Valster A, Tran NL, Nakada M, Berens ME, Chan AY, Symons M, Methods. 2005;37:208-215.

[34] Croce M, De Ambrosis A, Corrias MV, Pistoia V, Occhino M, Meazza R, Giron-Michel J, Azzarone B, Accolla RS, Ferrini S, Oncogene. 2003;22:7848-7857.
[35] Bellis SL, Miller JT, Turner CE, The Journal of biological chemistry. 1995;270:17437-17441.
[36] Kohn AD, Takeuchi F, Roth RA, Journal of Biological Chemistry. 1996;271:21920-21926.
[37] Reyland ME, Front Biosci. 2009;14:2386-2399.
[38] Johnson DB, Estrada MV, Salgado R, Sanchez V, Doxie DB, Opalenik SR, Vilgelm AE, Feld E, Johnson AS, Greenplate AR, Sanders ME, Lovly CM, Frederick DT, Kelley MC, Richmond A, Irish JM, Shyr Y, Sullivan RJ, Puzanov I, Sosman JA, Balko JM, Nat Commun. 2016;7.
[39] Jiang XF, Zhou J, Giobbie-Hurder A, Wargo J, Hodi FS, Clinical Cancer Research. 2013;19:598-609.
[40] Marzec M, Zhang Q, Goradia A, Raghunath PN, Liu XB, Paessler M, Wang HY, Wysocka M, Cheng MG, Ruggeri BA, Wasik MA, P Natl Acad Sci USA. 2008;105:20852-20857.
[41] Robbins PF, El-Gamil M, Li YF, Zeng G, Dudley M, Rosenberg SA, Journal of immunology. 2002;169:6036-6047.
[42] Altomonte M, Visintin A, Tecce R, Leonardi A, Calabro L, Fonsatti E, Pucillo C, Maio M, Journal of cellular physiology. 2004;200:272-276.
[43] Lehner M, Stockl J, Majdic O, Knapp W, Huttner K, Felzmann T, Holter W, Human immunology. 2003;64:762-770.
[44] Guan JL, Shalloway D, Nature. 1992;358:690-692.
[45] Persad S, Dedhar S, Cancer Metast Rev. 2003;22:375-384.
[46] Dissanayake SK, Olkhanud PB, O'Connell MP, Carter A, French AD, Camilli TC, Emeche CD, Hewitt KJ, Rosenthal DT, Leotlela PD, Wade MS, Yang SW, Brant L, Nickoloff BJ, Messina JL, Biragyn A, Hoek KS, Taub DD, Longo DL, Sondak VK, Hewitt SM, Weeraratna AT, Cancer research. 2008;68:10205-10214.
[47] Putnam AJ, Schulz VV, Freiter EM, Bill HM, Miranti CK, Cell Commun Signal. 2009;7:7-10.
[48] Lutticken C, Wegenka UM, Yuan JP, Buschmann J, Schindler C, Ziemiecki A, Harpur AG, Wilks AF, Yasukawa K, Taga T, Kishimoto T, Barbieri G, Pellegrini S, Sendtner M, Heinrich PC, Horn F, Science. 1994;263:89-92.
[49] Wang TH, Niu GL, Kortylewski M, Burdelya L, Shain K, Zhang SM, Bhattacharya R, Gabrilovich D, Heller R, Coppola D, Dalton W, Jove R, Pardoll D, Yu H, Nature medicine. 2004;10:48-54.
[50] Kitamura H, Kamon H, Sawa SI, Park SJ, Katunuma N, Ishihara K, Murakami M, Hirano T, Immunity. 2005;23:491-502.
[51] Chan LL, Cheung BK, Li JC, Lau AS, Journal of leukocyte biology. 2010;88:303-311.
[52] Boxhorn HK, Smith JG, Chang YJ, Guerry D, Lee WM, Rodeck U, Turka LA, Eck SL, Cancer immunology, immunotherapy : CII. 1998;46:283-292.
[53] Sakaguchi M, Oka M, Iwasaki T, Fukami Y, Nishigori C, J Invest Dermatol. 2012;132:1877-1885.

## Figure legends

**Fig. 1**: K**inetics of HLA-DRα and adhesion receptors expression in response to HLA-DR signalling.** The A375 and HT-144 melanoma cell lines were stimulated for 24h, 48h and 72h with the anti-HLA-DR antibody L243 or left unstimulated (NS). 40 μg of cell extracts were analysed through western blot experiments. The kinetics of (A) HLA-DRα molecules, (B) MCAM and (C) ICAM adhesion receptors was analysed in A375 cells (left panel) and in HT-144 cells (right panel) in response to HLA-DR signalling. The equal protein loading was confirmed by the analysis of the β-actin expression (A, B and C, lower panels). The data are representative of several western blot experiments performed with cellular lysates of almost three different experiments. The band

intensities of each western blot were quantified by densitometric analysis, are expressed as a per cent of unstimulated cells and are normalized for actin amount. The error bars indicate the standard deviation, statistical significance was analysed by the Student's t-test: *p < 0.05 was considered significant; **p < 0.01 highly significant; ***p < 0.001 very highly significant.

**Fig. 2: K**inetics of Integrins expression in response to HLA-DR signalling.** The A375 and HT144 melanoma cell lines were stimulated for 24h, 48h and 72h with the L243 antibody or left unstimulated (NS) and 40 μg of cell extracts were analysed through western blot experiments. kinetics of Integrin β□, Integrin β□ (A and B, high panels)□ Integrin α□ and Integrin α□ (A and B, middle panels) adhesion receptor expressions was analysed in A375 (A) and HT-144 (B) melanoma cells in response to HLA-DR signalling. The equal protein loading was confirmed by the analysis of the β-actin expression (A and B, lower panels). The data are representative of several western blot experiments performed with cellular lysates of almost three different experiments. The band intensities of each western blot were quantified by densitometric analysis, are expressed as a per cent of unstimulated cells and are normalized for actin amount. The error bars indicate the standard deviation, statistical significance was analysed by the Student's t-test: *p < 0.05 was considered significant; **p < 0.01 highly significant; ***p < 0.001 very highly significant.

**Fig. 3: HLA-DR signalling.** 40 μg of A375 and HT-144 melanoma cell extracts stimulated with the L243 antibody for 24h, 48h and 72h or left unstimulated (NS), were analysed through western blot experiments. The expression of (A) ILK (arrow), PAX and FAK, the phosphorylated form of FAK (pFAK) and PAX (pPAX) signalling molecules, the expression of (B) BRAF, ERK and the phosphorylated form of ERK (pERK) signalling molecules, were analysed in A375 (left panels) and HT-144 (right panels) in response to HLA-DR signalling. The equal protein loading was confirmed by the analysis of the β-actin expression (A and B, lower panels). The data are representative of several western blot experiments performed with cellular lysates of almost three different

experiments. The band intensities of each western blot were quantified by densitometric analysis, are expressed as a per cent of unstimulated cells and are normalized for actin amount.

**Fig. 4: HLA-DR signalling.** A375 and HT-144 melanoma cell extracts stimulated with the L243 antibody for 24h, 48h and 72h or left unstimulated (NS), were analysed through western blot experiments. The expression of (A) AKT and the phosphorylated form of AKT (pAKT) signalling molecules, (B) of PKCα and the phosphorylated forms of conventional and novel PKC isoforms (pPKCpan) signalling molecules, (C) of STAT3 and the phosphorylated form of STAT3 (pSTAT3) signalling molecules and (D) of PD-L1 receptor was analysed in A375 (left panels) and in HT-144 (right panels) melanoma cells in response to HLA-DR signalling. The equal protein loading was confirmed by the analysis of the β-actin expression (A, B, C and D, lower panels). The data are representative of several western blot experiments performed with cellular lysates of almost three different experiments. The band intensities of each western blot were quantified by densitometric analysis, are expressed as a per cent of unstimulated cells and are normalized for actin amount.

**Fig. 5: Lipid raft compartments of A375 cells in response to HLA-DR signalling.** A375 melanoma cells were stimulated for 24h (left panels) and 48h (right panels) with the L243 antibody or left unstimulated (NS) and the lipid raft compartments were isolated through discontinuous sucrose gradient. The localisation of (A) HLA-DRα, Integrin β1, Integrin β3, FAK, MCAM adhesion receptors and (B) STAT3, PD-L1 and AKT proteins on the low-density fractions (F2, F3 and F4) containing the lipid raft compartments was analysed through western blot experiments. The localisation on the isolated fractions of caveolin was analysed as a positive control of the raft compartments (A and B, lower panels). The data are representative of several western blot experiments performed with isolated lipid rafts of almost three different experiments. The band intensities (F2+F3+F4) of each western blot were quantified by densitometric analysis, are expressed as a per cent of unstimulated cells and are normalized for caveolin amount.

**Fig. 6: Lipid raft compartments of HT-144 cells in response to HLA-DR signalling.** HT-144 melanoma cells were stimulated for 24h (left panels) and 48h (right panels) with the L243 antibody or left unstimulated (NS) and the lipid raft compartments were isolated through discontinuous sucrose gradient. The localisation of (A) HLA-DRα, ICAM, Integrin β3, FAK proteins and (B) STAT3, PD-L1 and AKT proteins on the low-density fractions (F2, F3 and F4) containing the lipid raft compartments was analysed through western blot experiments. The localisation of the caveolin on the isolated fractions was analysed as a positive control of the raft compartments (A and B, lower panels). The data are representative of several western blot experiments performed with isolated lipid rafts of almost three different experiments. The band intensities (F2+F3+F4) of each western blot were quantified by densitometric analysis, are expressed as a per cent of unstimulated cells and are normalized for caveolin amount.

**Fig. 7: The HLA-DR signalling in MβCD induced lipid raft destruction of A375 melanoma cells.** The melanoma cells were treated for 24h and 48h with 1 mM MβCD (MβCD), or stimulated with the L243 antibody (L243), or stimulate and treated with the same amount of L243 and MβCD (L243+MβCD), or left unstimulated (NS). The expression of (A) HLA-DRα, (B) MCAM, Integrin β1 and Integrin β3 adhesion receptors, (C) FAK and STAT3 signalling molecules, PD-L1 receptor and the phosphorylated form of FAK (pFAK) was analysed through western blot experiments. The equal protein loading was confirmed by the analysis of the β-actin expression (A, B and C, lower panels). The data are representative of several western blot experiments performed with cellular lysates of almost three different experiments. The band intensities of each western blot were quantified by densitometric analysis, are expressed as a per cent of unstimulated and untreated cells and are normalized for actin amount.

**Fig. 8: The HLA-DR signalling in MβCD induced lipid raft destruction of HT-144 melanoma cells.** The melanoma cells were treated for 24h and 48h with 1 mM MβCD (MβCD), or stimulated with the L243 antibody (L243), or stimulate and treated with the same amount of L243 and MβCD (L243+MβCD), or left unstimulated (NS). The expression of A) HLA-DRα, (B) ICAM, Integrin β1 and Integrin β3 adhesion receptors, (C) FAK and STAT3 signalling molecules, PD-L1 receptor as well as the phosphorylated form of FAK (pFAK) was analysed through western blot experiments. The equal protein loading was confirmed by the analysis of the β-actin expression (A, B and C, lower panels). The data are representative of several western blot experiments performed with cellular lysates of almost three different experiments. The band intensities of each western blot were quantified by densitometric analysis, are expressed as a per cent of unstimulated and untreated cells and are normalized for actin amount.

**Fig. 9: The HLA-DR signalling increases the migration and invasion of melanoma cell.** A375 and HT-144 human melanoma cells, L243 stimulated (L243) for 32h or unstimulated (NS) were plated in the inner chamber of a 24-well culture plate with PET membrane (8μm pore size) (A, middle and left panels) and incubated at 37°C for 16h. A375 melanoma cells, L243 stimulated (L243) for 26h or unstimulated (NS), were plated in the inner chamber of a 24-well culture plate with PET membrane coated with a matrigel layer (A, right panel) and incubated at 37°C for 22h. The migrated cells fixed and stained, were counted under phase contrast microscope (Leica) and the stimulated migrating cells, are expressed as a per cent of unstimulated migrating cells. Confluent A375 (B, left panels) and HT-144 (B, right panels) melanoma cells were scratch-wounded and L243 stimulated (L243) or unstimulated (NS) for 48h at 37°C. The images of wound closure were visualised by inverted phase contrast microscopy (Leica) and were captured using digital imaging at 0h (T0), 24h (T1) and 48h (T2) (B, left and right panels), the areas free of stimulated cells at T1 and T2 are expressed as a per cent of wound closure compared with areas free of cells at T0 (B, higher panels); the areas free of stimulated cells at T2 are expressed also as a per cent of wound closure

compared with areas free of unstimulated cells at T2 (B, middle panels). The data are representative of almost three different experiments, the error bars indicate the standard deviation and statistical significance was analysed by the Student's t-test: *$p < 0.05$ was considered significant; **$p < 0.01$ highly significant; ***$p < 0.001$ very highly significant.

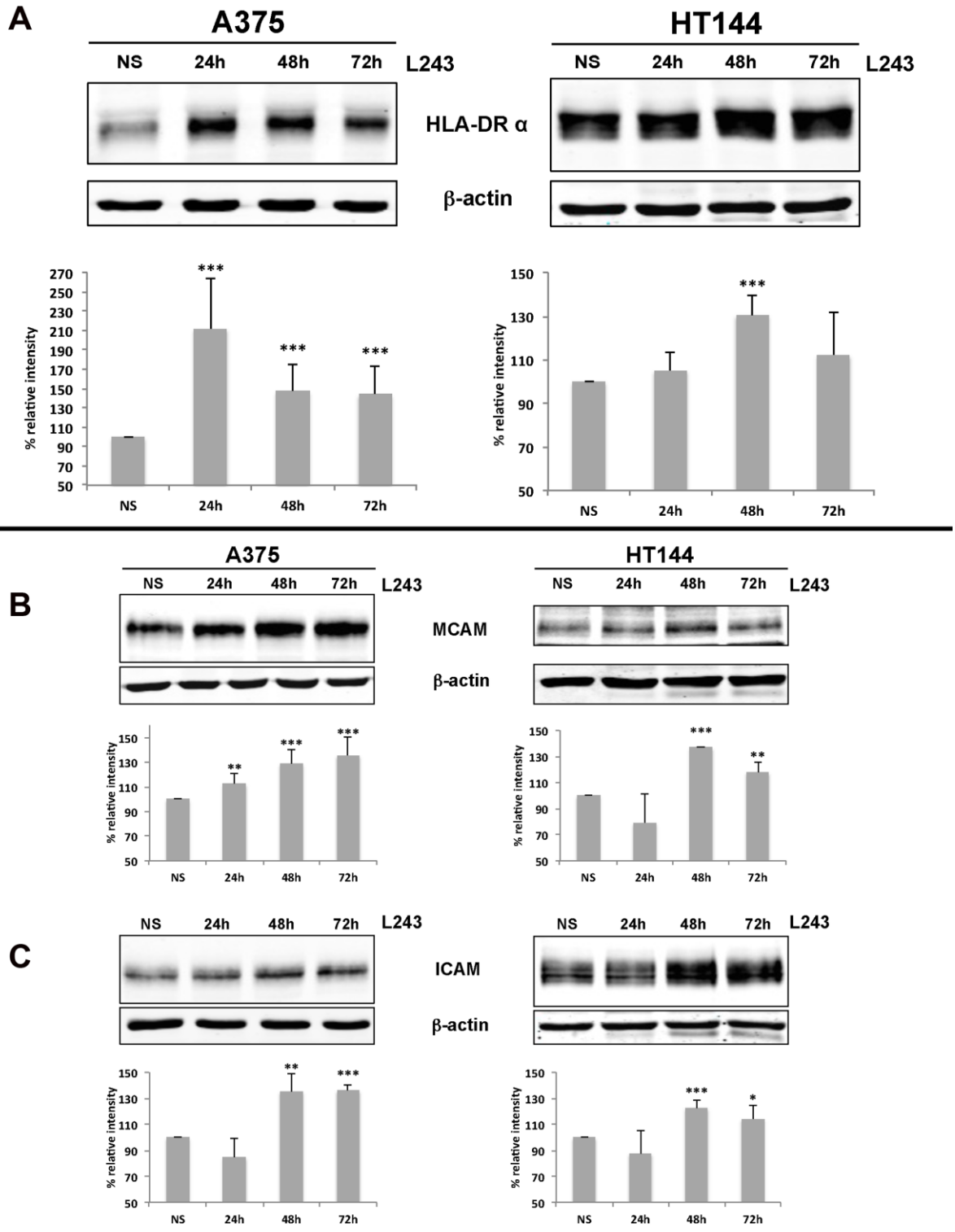



Fig. 1

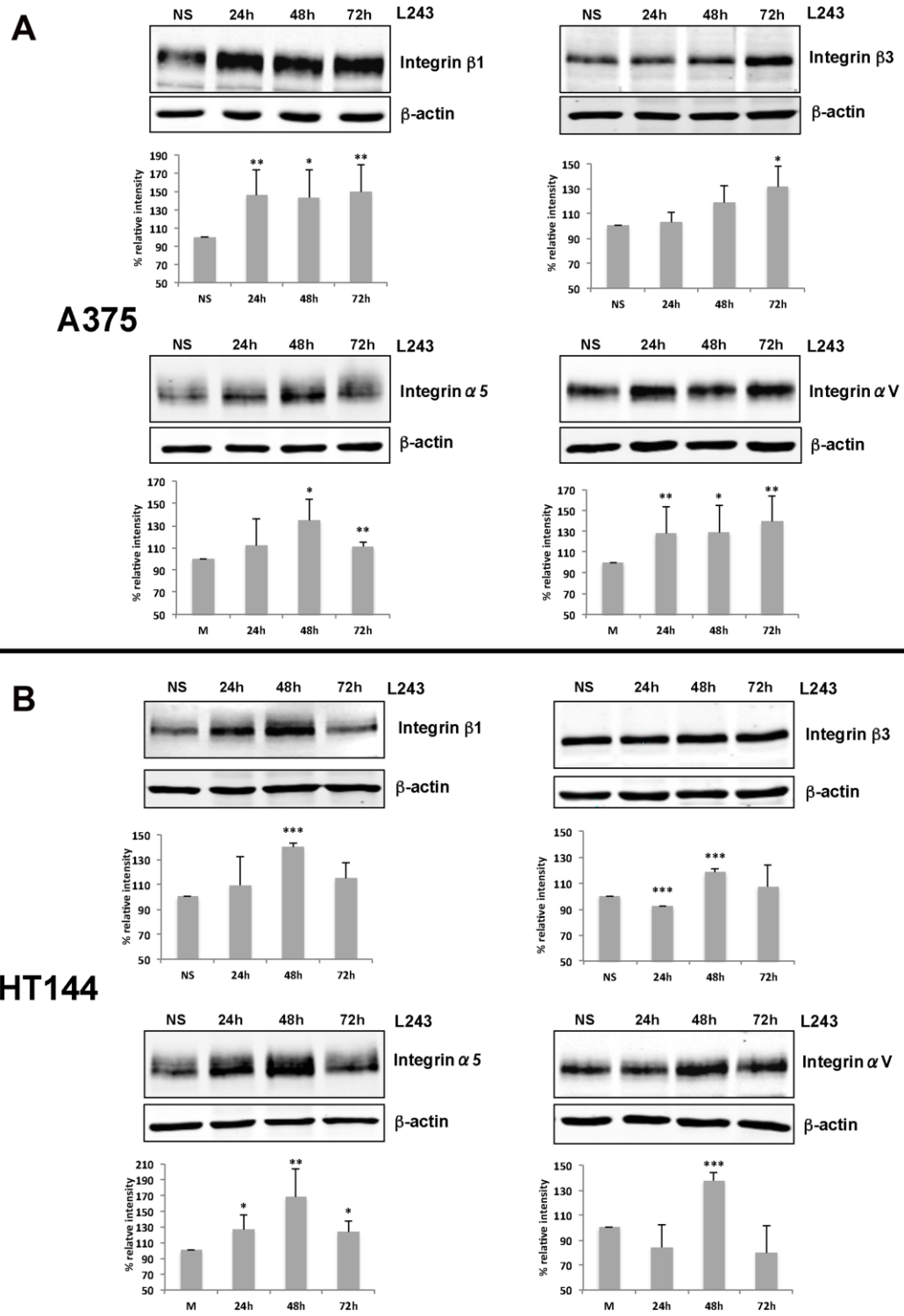
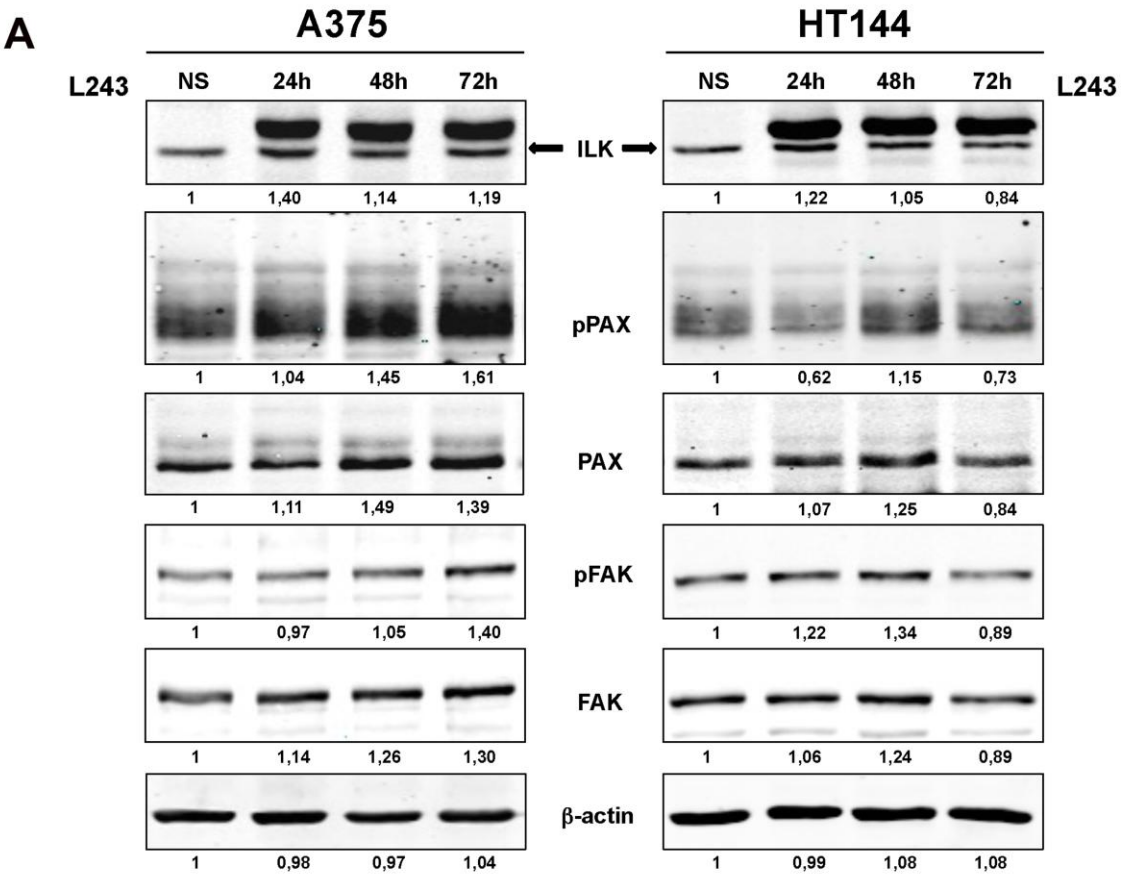



Fig. 2



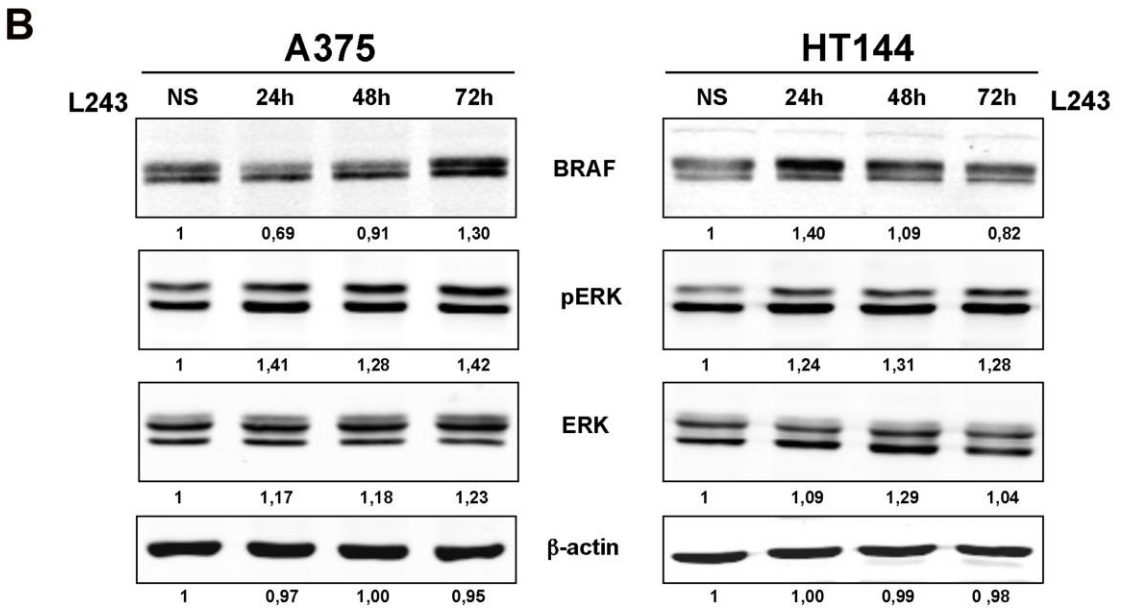




Fig. 3

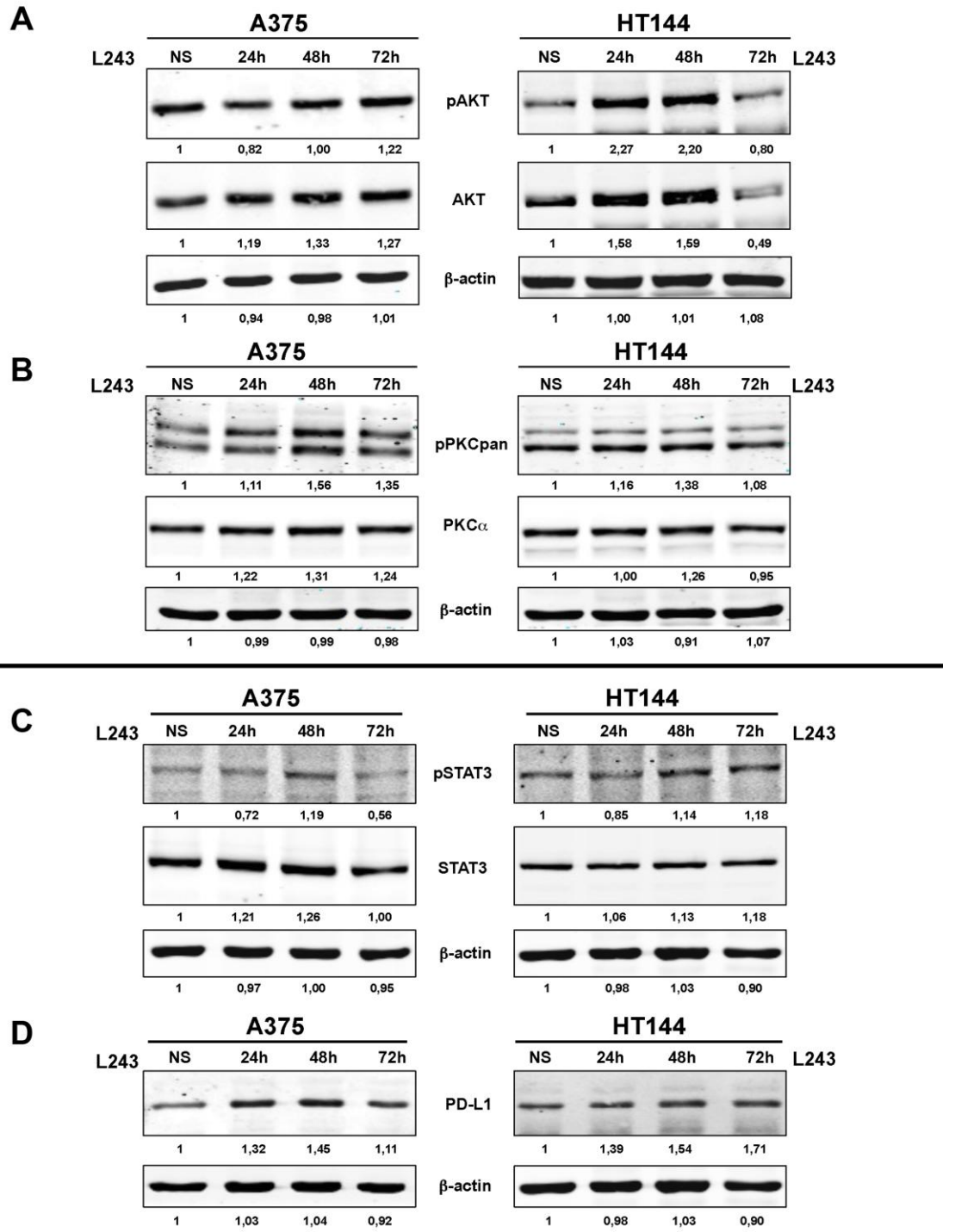



Fig. 4

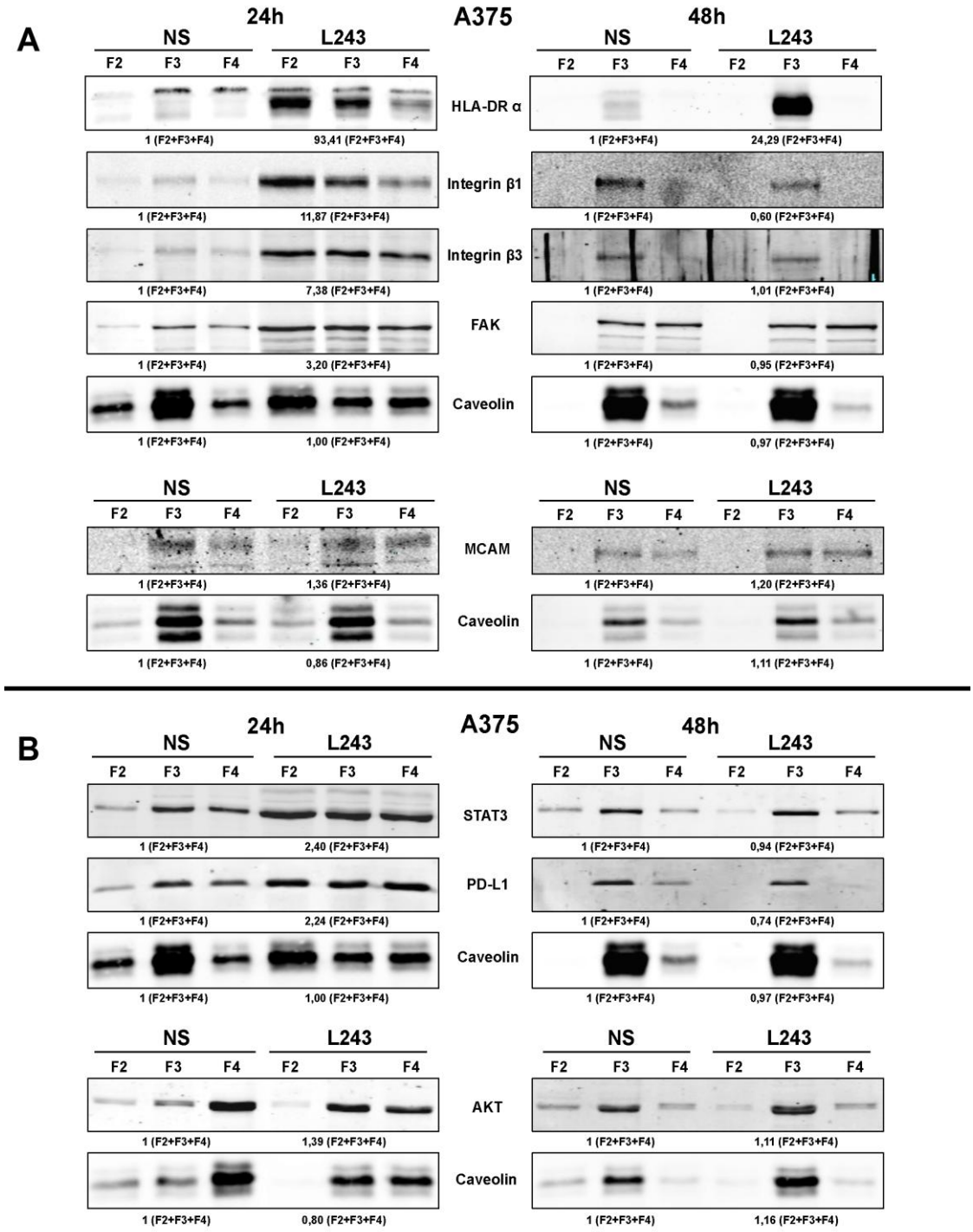



Fig. 5

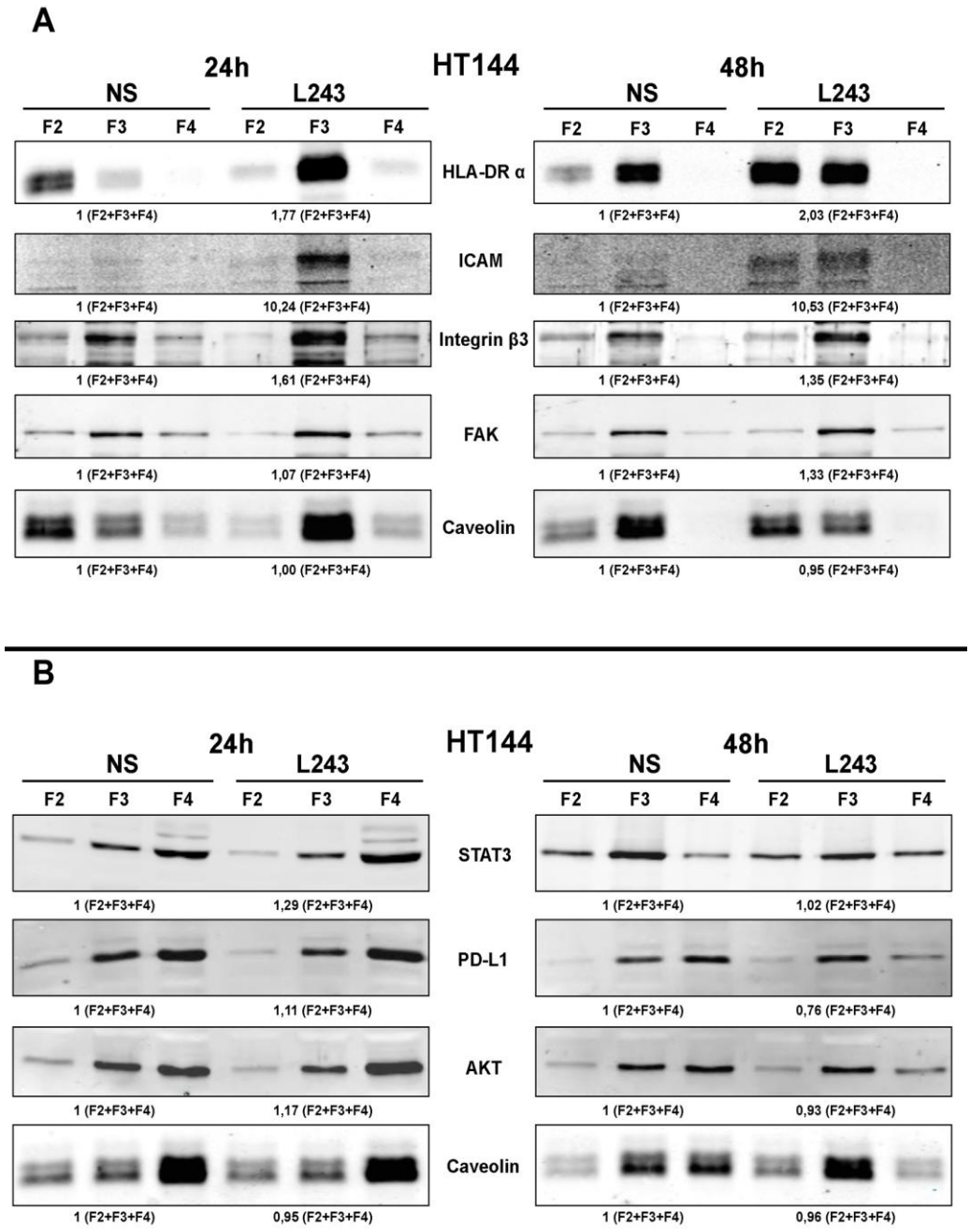



Fig. 6

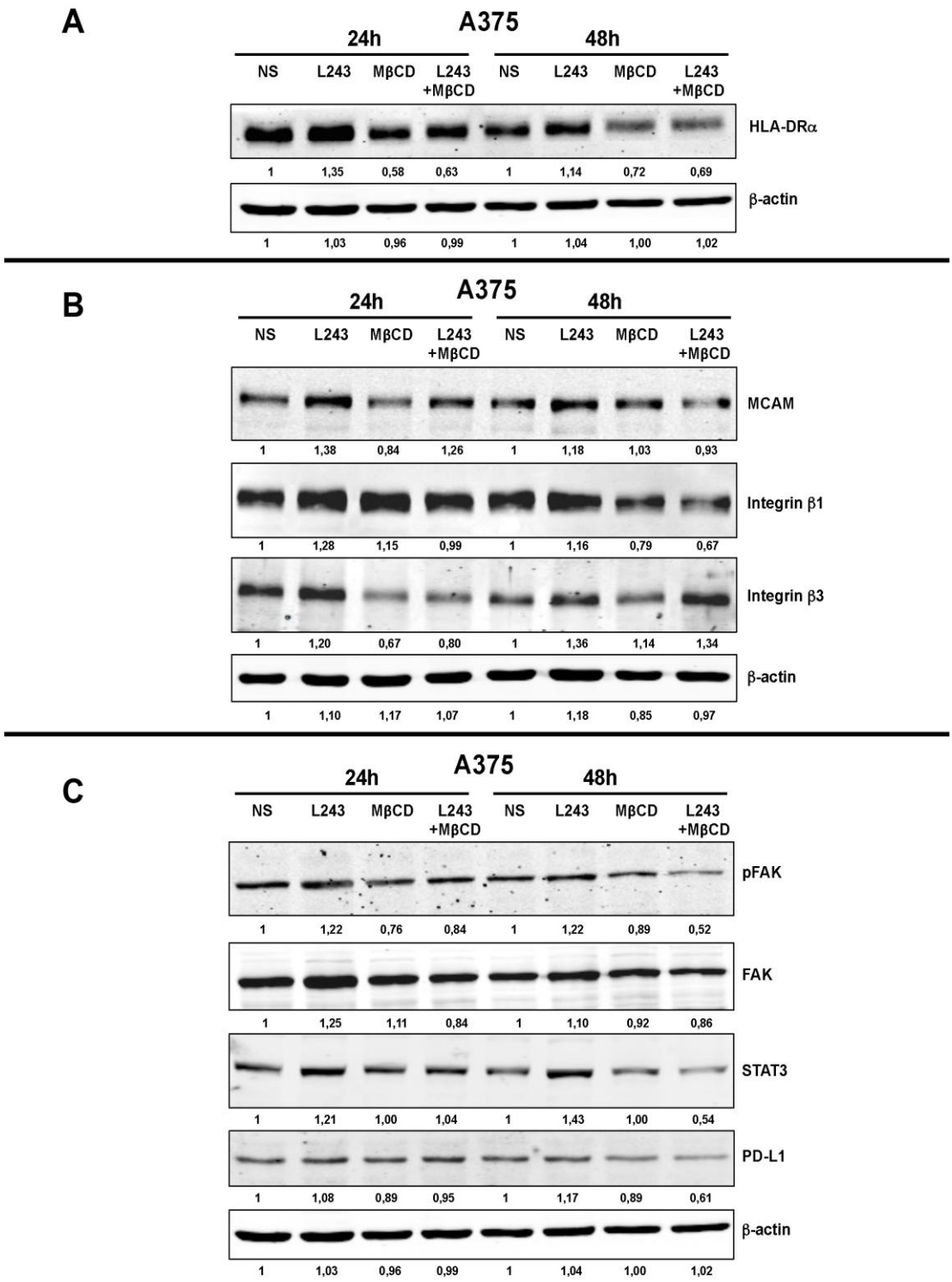



Fig. 7

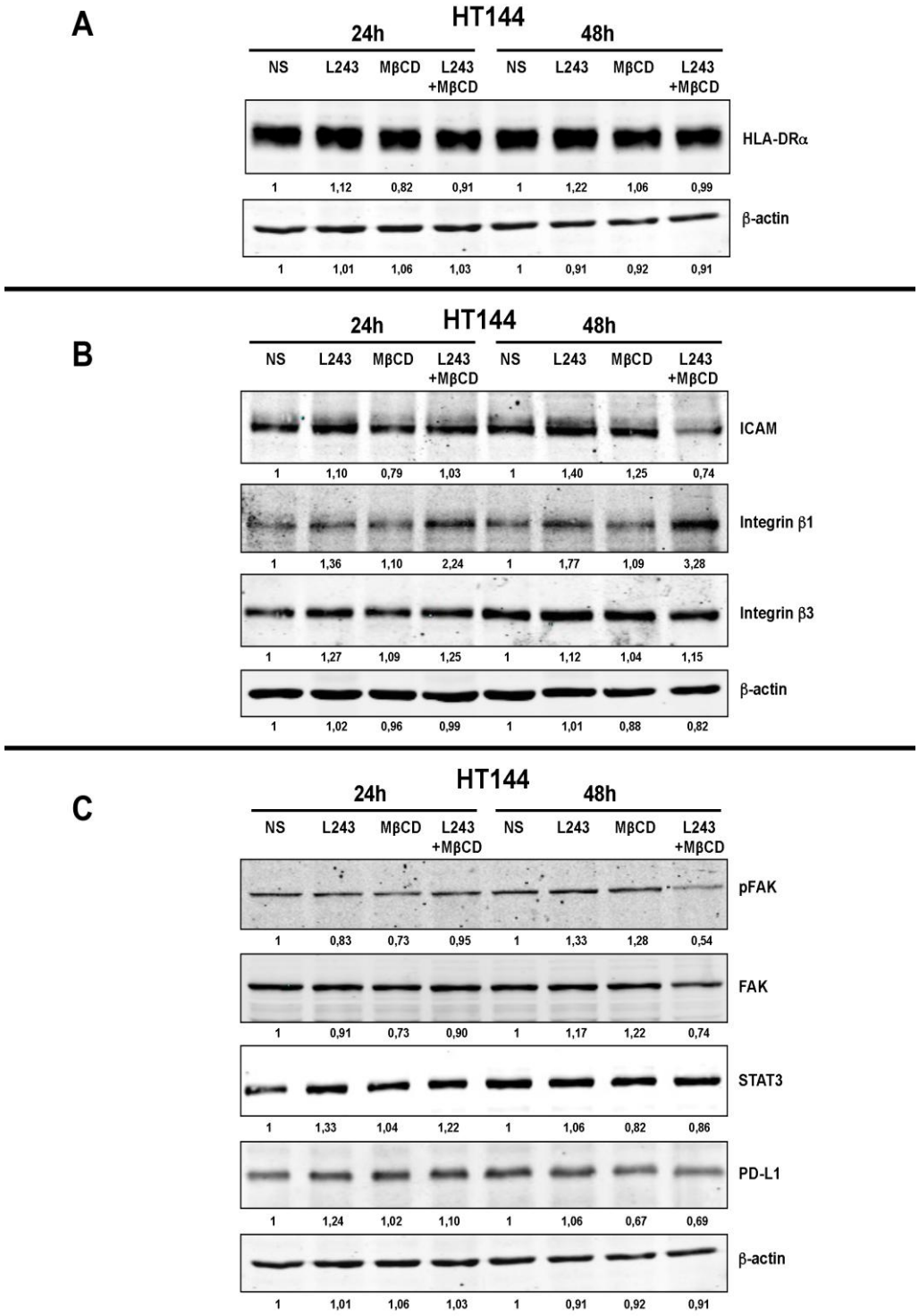



Fig. 8

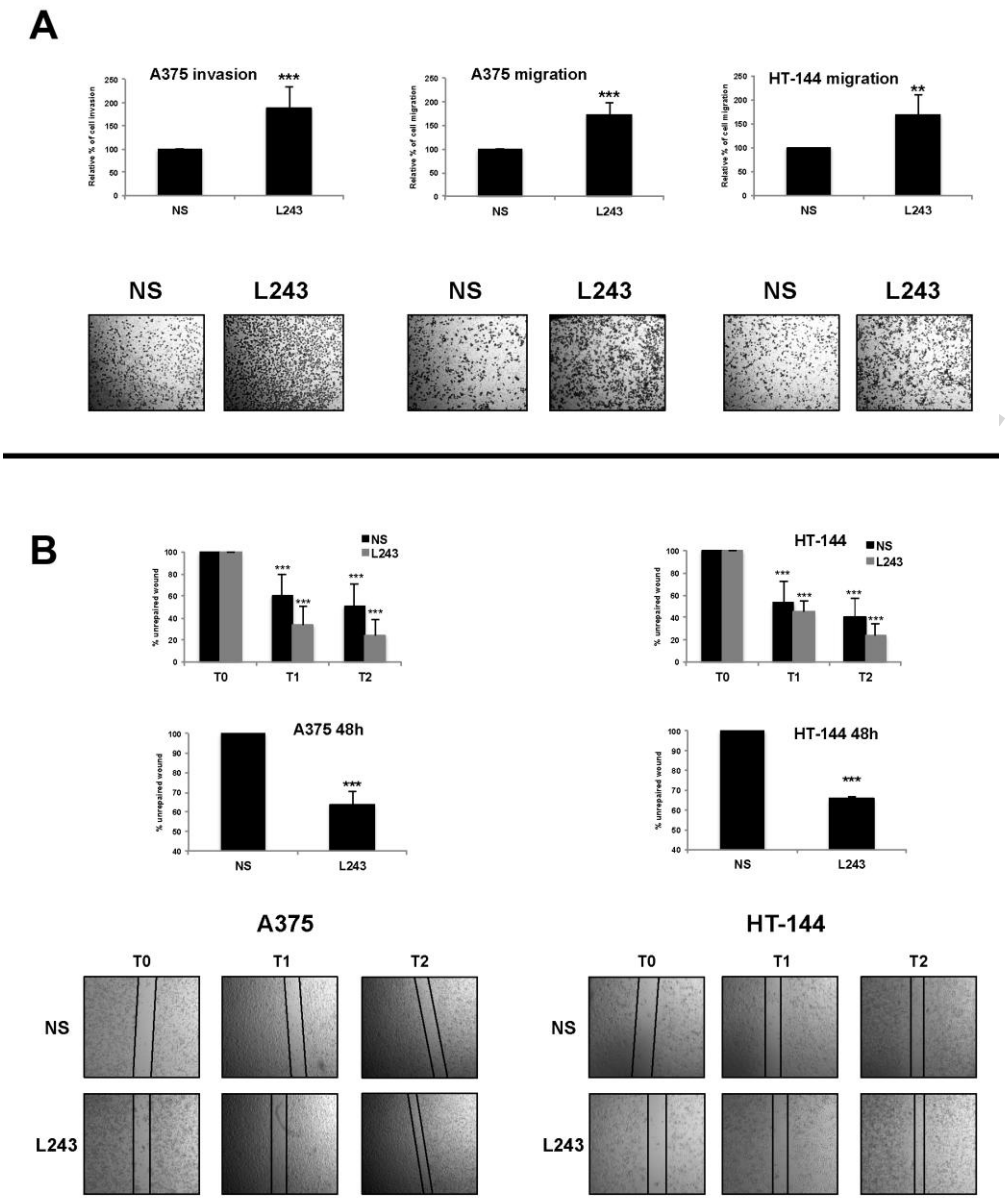



Fig. 9

# The HLA-DR mediated signalling increases the migration and invasion of melanoma cells, the expression and lipid raft recruitment of adhesion receptors, PD-L1 and signal transduction proteins

Francesca Costantini and Giovanna Barbieri

## Highlights

- Constitutive expression of MHC class II in melanoma is associated to bad prognosis.
- The consequence of HLA-DR signalling is adhesion receptors, PD-L1 and signalling proteins increased expression.
- HLA-DR signalling increases lipid raft recruitment of adhesion receptors, PD-L1 and signalling proteins.
- In melanoma cells, the HLA-DR signalling mediates the activation of several signalling pathways.
- The HLA-DR signalling increases *in vitro* migration and invasion of melanoma cells.
- Targeting HLA-DR mediated signalling could inhibit melanoma progression and immune escape.