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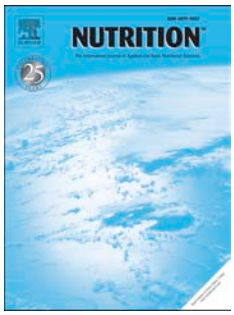

Capsaicin-Mediated Apoptosis Of Human Bladder Cancer Cells Activates Dendritic Cells Via Cd91

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CAPSAICIN-MEDIATED APOPTOSIS OF HUMAN BLADDER CANCER CELLS ACTIVATES DENDRITIC CELLS VIA CD91

Running title: DC activation by capsaicin

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Abbreviations: capsaicin (CPS), dendritic cells (DCs), immature dendritic cells (iDC), immunogenic cell death (ICD), damage-associated molecules (DAMPs), pattern recognition receptors (PRRs), calreticulin (CRT), heat-shock protein (HSP), vanilloid subfamily member 1 (TRPV1), capsazepine (CPZ).

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Abstract

Objectives: Immunostimulation by anticancer cytotoxic drugs is needed for long-term therapeutic success. Activation of dendritic cells (DCs) is crucial to obtain effective and long-lasting anticancer T cell-mediated immunity. The aim of this study was to explore the effect of capsaicin-mediated cell death of bladder cancer cells on the activation of human monocyte-derived $CD1a^+$ immature DCs.

Methods: Immature DCs, generated from human peripheral blood-derived $CD14^+$ monocytes cultured with granulocyte-macrophage colony stimulating factor and interleukin-4, were cocultured with capsaicin (CPS)-induced apoptotic bladder cancer cells. DC activation was investigated using immunofluorescence and flow cytometric analysis for key surface molecules. In some experiments, CD91 was silenced in immature DCs.

Results: We found that capsaicin-mediated cancer cell apoptosis upregulates CD86 and CD83 expression on DCs, indicating the induction of DC activation. Moreover, silencing of CD91 (a common receptor for damage-associated molecular patterns, such as calreticulin and heat-shock protein-90/70) in immature DCs, led to the inhibition of DC activation.

Conclusions: Our data show that CPS-mediated cancer cell apoptosis activates DCs via CD91, suggesting CPS as an attractive candidate for cancer therapy.

Keywords: Capsaicin, Apoptosis, Dendritic cells, CD91, bladder cancer

Introduction

Evidence exists that combinatorial anticancer therapeutic interventions are needed to completely eradicate neoplastic lesions and that the functional state of the host immune system has a major role in long-term therapeutic success [1-3]. Thus, great interest has been attracted by anticancer immunochemotherapy, aimed at combining chemotherapy-induced cancer cell cytotoxicity with antitumor immune responses to achieve complete and long-lasting removal of cancer cells [3]. The activation of dendritic cells (DCs) is an absolute prerequisite for the stimulation of effective and long-lasting T-cell mediated anticancer immune response [2]. Today, a rather restrict number of conventional chemotherapeutics are known to activate DCs by inducing a peculiar form of cancer cell apoptosis with immunogenic properties, denominated immunogenic cell death (ICD) [3]. The latter relies on the spatiotemporal-defined (i.e. pre/early- and late-apoptotic) emission by dying cancer cells of a certain number of damage-associated molecules (DAMPs), which operate on pattern recognition receptors (PRRs) expressed by DCs [3]. In particular, the emission of chaperones, including calreticulin (CRT) [4], heat-shock protein (HSP) 90 [5] and HSP 70 [6], has been indicated as one of the most important mechanism for the activation of DCs. Recently, Cirone et al. [7] has shown a pivotal role of CD91, a common receptor for CRT and HSP90/70, in DC activation by lymphoma cell death induced by cytotoxic drugs such as Bortezomib and Tyrphostin AG 490.

Capsaicin (CPS) (*trans*-8-methyl-*N*-vanillyl-6-nonenamide), a unique alkaloid found primarily in the fruit of the plant genus *Capsicum*, is the main ingredient responsible for the hot pungent taste of chili peppers [8]. It exerts various beneficial effects on different human diseases, including many types of cancer [9,10]. The mechanism of action of CPS has been extensively studied over the past decade. CPS binds to the transient receptor potential vanilloid subfamily member 1 (TRPV1), predominantly expressed by sensory neurons, but also expressed by a variety of other normal

(including inflammatory cells) and neoplastic cells [9,10]. However, it has been well established that CPS can acts on several different cells in a TRPV1-independent manner [9,10]. Intriguingly, although we possess limited and rather controversial data on both TRPV1 expression and CPS function on mouse bone marrow- [11,12] and human monocyte-derived DCs [13], it has been shown that intratumoral administration of CPS leads to the regression of advanced preexisting tumors by stimulating anticancer T cell-mediated immune response [14,15]. Moreover, it is well known that CPS induces cell death in a variety of cancer cells [9,10]. In this regard, we have recently shown that CPS-mediated apoptosis of human bladder cancer cell lines leads to the emission of DAMPs of ICD by cancer cells, including CRT, HSP90 and HSP70 [16]. In the present study we investigated whether CPS-mediated apoptosis of bladder cancer cells leads to the activation of human monocyte-derived DCs and whether CD91, a common CRT and HSP 90/70 receptor, is involved in this process.

Materials and methods

Cell lines, treatments and apoptosis

T24 and SD48 human urinary bladder cancer cell lines were induced to undergo apoptosis by CPS (Sigma-Aldrich, St. Louis, USA) as previously described [16]. Apoptosis was assessed by annexin V-FITC and propidium iodide staining [16].

Immature DC (iDC) generation

Human peripheral blood mononuclear cells from healthy donors were isolated by Fycoll-Paque gradient centrifugation (Pharmacia, Uppsala, Sweden). Monocytes were isolated by immunomagnetic cell separation using anti-CD14-conjugated microbeads (Miltenyi Biotec, Italy).

To induce the differentiation of iDCs, monocytes were cultured for 6 days with recombinant human granulocyte-macrophage colony stimulating factor (50 ng/ml) and interleukin-4 (20 ng/ml) (Miltenyi Biotec) as described [7].

iDC treatment with CPS

iDCs were incubated with the indicated CPS doses for 24 h, at 37°C. Lipopolysaccharide (LPS)-stimulated DCs were used as positive control. In some experiments iDCs were pretreated with 10 μM capsazepine (CPZ) (Sigma) for 1 h, at 37°C.

iDC/tumor cell cocultures

iDCs were cocultured with CPS-treated tumor cells for 48 h, at a 1:2 iDC/tumor cell ratio. In some experiments iDCs were transfected with CD91siRNA.

Flow cytometric analysis

Immunofluorescence was performed using appropriate isotype control antibodies or antibodies against CD1a (BD Biosciences Pharmingen), CD86 and CD83 (Miltenyi Biotec) or CD91 (Santa Cruz Biotecnology). Then, cells were analyzed by FACSCalibur, using CELLQuest software (BD Biosciences, San Jose, USA) [16]. DCs were gated according to their FSC and SSC properties. At least 5 x 10^3 events were acquired for each sample.

Western blotting

Proteins were separated by SDS-PAGE, blotted into nitrocellulose (Schleicher & Schuell BioScience GmbH, Dassel, Germany) and incubated with antibodies against CD91 (Santa Cruz), TRPV1 or β actin (both from Sigma). The reaction was revealed by horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories, USA) and detected by ECL (Amersham, Piscataway, USA).

CD91 silencing

iDCs were transfected with CD91siRNA or with non-targeting siRNA (siCONTROL) (Santa Cruz Biotecnology) using INTERFERin® siRNA Transfection Reagent (Polyplus Transfection, USA), according to manufacture's instructions.

Statistical analysis

Student's *t* test was used for all analyses; $p<0.05$ was considered significant.

Results

Activation of human monocyte-derived DCs by CPS

Taking into consideration the contrasting results in the literature [11-13] on the ability of CPS to directly affect the activation of DCs, before investigating the effect of CPS-mediated cancer cell apoptosis on DC activation, we analyzed TRPV1 expression and CPS function on monocyte-derived $CD1a^+$ iDCs [17]. We observed that $CD1a^+$ iDCs (Fig. 1a, left panel) expressed TRPV1 at the protein level (Fig.1a, right panel). Moreover, the exposition of iDCs to increasing concentrations (from 10 to 250 μM) of CPS for 24 h, at 37°C, induced a dose-dependent upregulation of CD86 costimulatory molecule and a slight but significant induction of $CD83^+$ maturation marker on viable DCs cells (Fig.1b). Furthermore, the addition of CPZ, the TRPV1 antagonist, partially inhibited both CD86 and CD83 enhancement by CPS (Fig.1b). These results indicate that CPS is directly sensed by iDCs and that this sensitivity is partially dependent on TRPV1. Moreover, we found (data not shown) that CPS exerted a dose-dependent cytotoxic effect on DCs, in that 25% and 50% dead cells were found when iDCs were treated with 150 and 250 μM CPS, respectively, doses generally required to induce apoptosis in a variety of cancer cells [9,16].

To investigate the ability of cancer cell apoptosis mediated by CPS to lead to DC activation,

T24 and SD48 human bladder cancer cell lines were induced to undergo apoptosis by CPS [16]. We have previously shown that CPS-induced apoptotic T24 and SD48 cells emit all known DAMPs of ICD in the proper spatiotemporal-defined combination, including both the pre/early apoptotic plasma membrane exposure and the late-apoptotic extracellular release of CRT, HSP70 and HSP90 [16]. Thus, early- (30 and 12 hours) and late-apoptotic (96 hours) T24 (Fig. 1c, left panels) and SD48 (Fig. 1d, left panels) cancer cells [16], following extensive washes (to wash out CPS from medium), were cocultured with *in vitro* generated $CD1a^+$ iDCs. After 48 hours, DC activation was investigated by cytofluorimetric analysis of CD86 and CD83 cell surface expression. As illustrated in Fig. 1c and d, right lower panels, substantial upregulation of CD86 and significant induction of CD83 were found in DCs cocultured with 96 hour-CPS-treated T24 and SD48 cells, compared to 96 hour-vehicle-treated cells (Ctr). Interestingly, DC activation, despite to a lesser extent, was also induced by early-apoptotic cancer cells, that is 30 hour- and 12 hour-CPS-treated T24 and SD48 cells, respectively (Fig.1c and d, right upper panels). These findings indicate that CPS activates DCs by promoting apoptosis of cancer cells.

Inhibition of DC activation by CD91 silencing

To investigate whether DC activation is due to the emission of DAMPs of ICD by CPS-mediated dying cancer cells, we examined a possible role of CD91 (a common receptor for CRT and HSP90/70) in this process. To this end, we downregulated CD91 expression in CD1a+ iDCs by CD91 silencing, using CD91siRNA. The silencing efficacy at 24 hours was analyzed by both Western blotting in iDC lysates and immunofluorescence on iDC surface. We found that transfection of iDC with CD91siRNA, but not with siCONTROL, noticeably reduced both total (Fig. 2a) and cell surface (Fig. 2b) CD91 protein levels. The analysis of the activation capability of CPS-treated T24 cells on siRNA-transfected iDCs, showed the inhibition of both CD86 and CD83 markers in CD91-targeted cells compared to cells transfected with siCONTROL (Fig. 2c). These results show that CD91 silencing in DCs inhibits their activation.

Discussion

DCs are known to play a pivotal role in the induction of tumor antigen-specific T cells and thus of effective long-lasting antitumor immunity [2]. Therefore, DC activation by cytotoxic drug is essential for long-term therapeutic success [1, 3].

Our findings show that CPS activates DCs by eliciting cancer cell apoptosis. Interestingly, DC activation correlated with the emission by cancer cells of the spatiotemporal-defined combination of ICD determinants [3,16]. In fact, the activation of DCs was observed not only when iDCs were cocultured with late-apoptotic cancer cells (that is when damaged cells passively release DAMPs into the extracellular space), but also with early-apoptotic cancer cells, corresponding to cells actively exposing CRT and HSP90 on their plasma membrane [3,16].

We also demonstrated a role for CD91, the common receptor for CRT and HSP-90/70, in the activation of DCs by CPS-mediated cancer cell apoptosis. This finding is important, since CD91 ligation, at early-apoptosis, of cell surface exposed CRT and HSP90 results in “eat-me” signals to DCs [3-5], promoting the engulfment of cancer cell apoptotic corpses. Then, CD91 ligation, at late-apoptosis, of extracellular HSPs (usually formed of tumor antigen-HSP complexes) promotes the capture of tumor antigens by DCs, leading to a more efficient antigen processing than that obtained with antigen alone [3, 6]. These events are crucial for T cell cross-priming by DCs and thus for the stimulation of effective long-lasting antitumor immunity.

Finally, concerning the debated possible direct effect of CPS on DCs, according to Basu and Srivastava [11] and to Tóth et al. [13], we observed that iDCs express TRPV1 and, according to Basu and Srivastava [11], we found a direct activation of iDCs by CPS. However, it is important to consider, that CPS, used at doses generally required to induce apoptosis in cancer cells [9,16], exerted toxic effect on iDCs. This finding might be consistent with the observations by Gosh and Basu [18], describing a modification of the tumor microenvironment by *in vivo* administration of

CPS, consisting in stromal destruction and depletion of suppressive macrophages. DCs are heterogeneous in term of their nature and evolution (i.e. immunogenic *vs* tolerogenic) of their maturation state [2]. Therefore, different maturation states of DCs might be differently affected by different CPS doses and exposition times. More in-depth *in vitro* and *in vivo* studies are needed to ascertain and clarify the direct activity of CPS on DCs.

Conclusions

In conclusion, we provide evidence that cancer cell apoptosis mediated by CPS activates human DCs and that DC activation is mediated through CD91. Although further studies are needed to clarify the direct activity of CPS on DCs, CPS-mediated tumor cell death associated to DC activation could make CPS an attractive candidate for cancer therapy.

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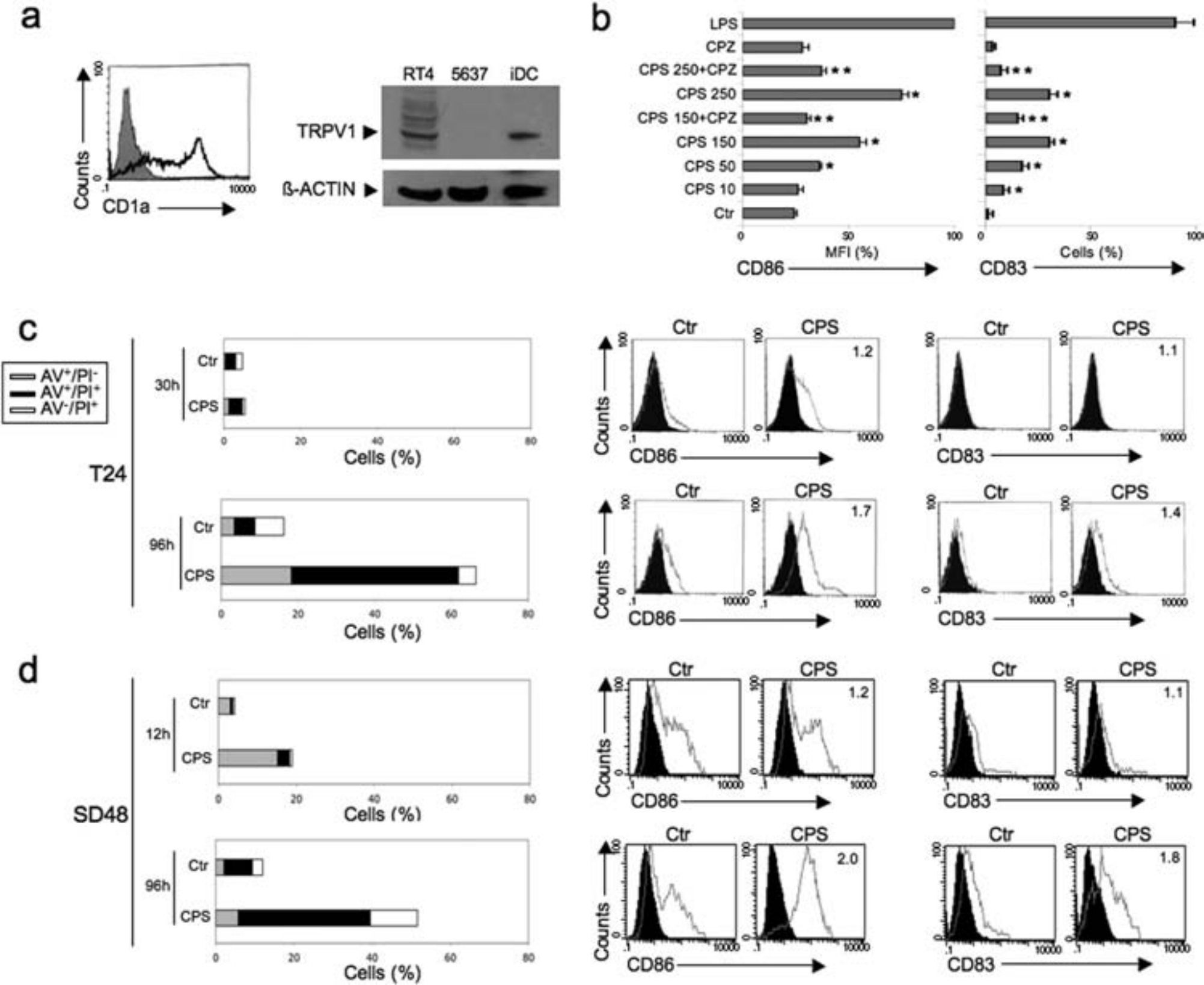
LEGENDS TO FIGURES

Fig. 1. Effect of CPS on DC activation. (*a, left panel*) Flow cytometry for CD1a expression in human monocyte-derived iDCs [17]; staining with isotype control (filled histogram) is also shown. (*a, right panel*) Western blotting for TRPV1 expression by iDCs; RT4 and 5637 human bladder cancer cell lines were used as positive and negative controls, respectively [19]; β-actin was used as loading control. (*b*) Flow cytometry for CD86 (expressed as percentage of MFI-Mean Fluorescence Intensity) and CD83 expression on DCs incubated with vehicle (Ctr) or with the indicated CPS doses (μM); LPS-treated DCs were used as positive control; in some experiments cells were pretreated with the TRVP1 agonist capaszepine (CPZ); data are represented by the mean ± S.D. of three independent experiments; *$p<0.05$ *vs* Ctr; ** $p<0.05$ *vs* CPS-treated cells. (*c and d, right panels*) Flow cytometry for CD86 and CD83 expression (numbers indicate MFI ratio, between the MFI of positive cells and MFI of control) on DCs cocultured with (*c and d, left panels*) vehicle (Ctr) or CPS-treated T24 and SD48 cancer cells undergoing apoptosis [16]; apoptosis, expressed as annexin V-FITC (AV) and propidium iodide (PI) cell staining. In *c and d, right panels*, staining with isotype control (filled histograms) is also shown; representative experiment out of three.

Fig. 2. Effect of CD91 silencing on DC activation by CPS. CD91siRNA-mediated downregulation was verified at 24 hours by: (*a*) western blotting for CD91 expression in iDC lysates; β-actin was used as loading control; numbers indicate the CD91siRNA/siCONTROL ratio intensities of bands, measured using Quantity One 1-D Analysis software; and (*b*) flow cytometry for CD91 expression by siCONTROL-(dark grey line) and CD91siRNA-(light grey line)-transfected iDCs. *(c*) Flow cytometry for CD86 and CD83 markers in siCONTROL-(dark grey line) and CD91siRNA-(light grey line)-transfected DCs cocultured with CPS-treated T24 cancer cells; staining with isotype control (filled histograms) is also shown. Representative experiment out of three.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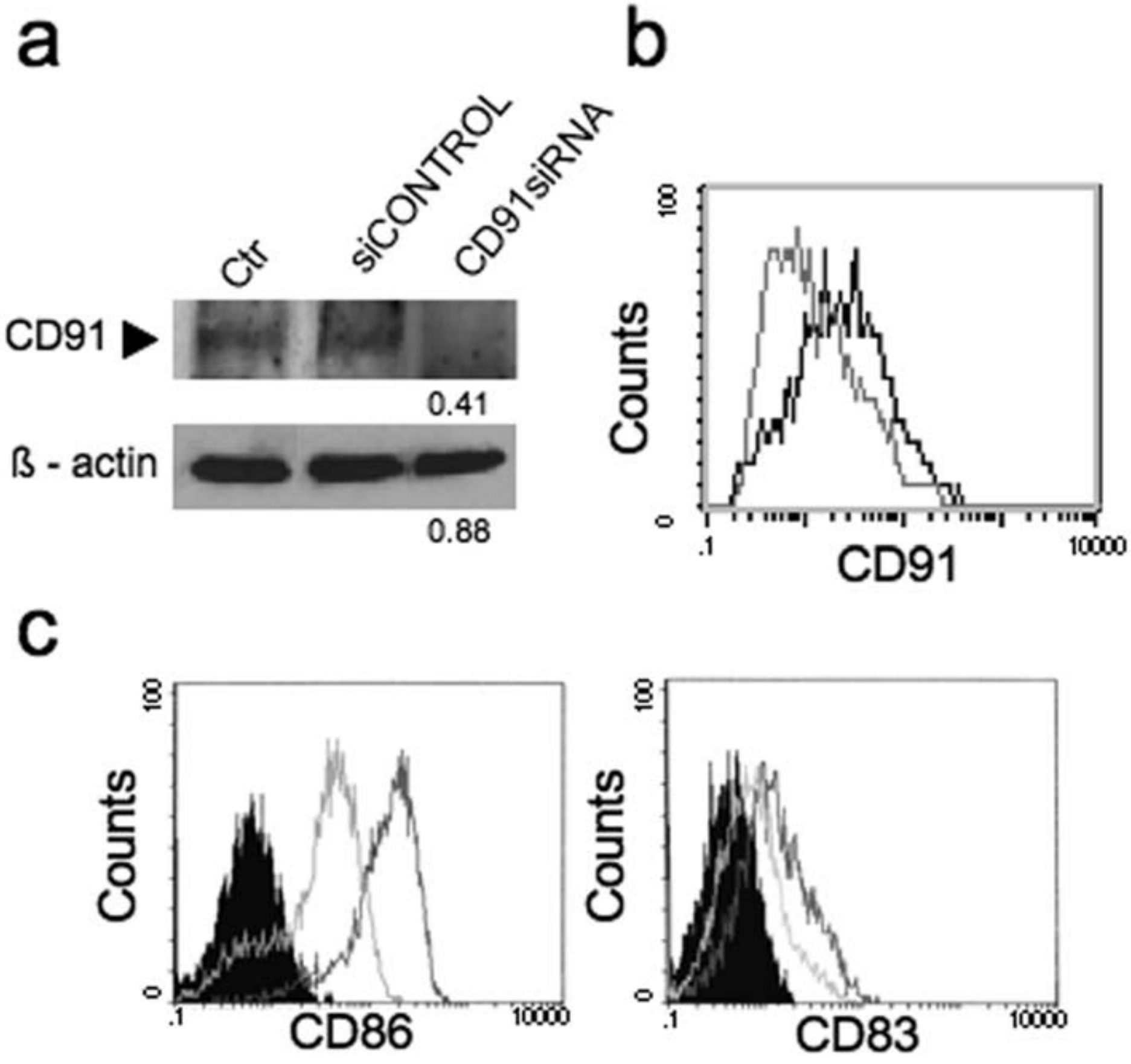
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.Capsaicin activates dendritic cells by inducing cancer cell apoptosis.

.Activation of dendritic cells by capsaicin-mediated cancer cell death occurs via CD91 (a common receptor of calreticulin (CRT), heat shock protein (HSP)-90 and -70).

.Capsaicin is an attractive candidate for cancer therapy since most conventional anticancer cytotoxic drugs fail to elicit activation of dendritic cells through induction of cancer cell apoptosis.

.Capsaicin might be used as adjuvant in bladder cancer therapy, since urinary bladder is one anatomic region suitable for local/intravesical therapy.