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Dopamine inhibits human CD8+ Treg function through D_1-like dopaminergic receptors

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ABSTRACT

CD8+ T regulatory/suppressor cells (Treg) affect peripheral tolerance and may be involved in autoimmune diseases as well as in cancer. In view of our previous data showing the ability of DA to affect adaptive immune responses, we investigated the dopaminergic phenotype of human CD8+ Treg as well as the ability of DA to affect their generation and activity. Results show that CD8+ T cells express both D_1-like and D_2-like dopaminergic receptors (DR), tyrosine hydroxylase (TH), the rate-limiting enzyme in the synthesis of DA, and vesicular monoamine transporter (VMAT) 2 and contain high levels of intracellular DA. Preferential upregulation of DR mRNA levels in the CD8+CD28- T cell compartment occurs during generation of CD8+ Treg, which is reduced by DA and by the D_1-like DR agonist SKF-38393. DA and SKF-38393 also reduce the suppressive activity of CD8+ Treg on human peripheral blood mononuclear cells. Treg are crucial for tumor escape from the host immune system, thus the ability of DA to inhibits Treg function supports dopaminergic pathways as a druggable targets to develop original and innovative antitumor strategies.

1. Introduction

Dopamine (DA), a key neurotransmitter in the brain and in peripheral tissues, has been increasingly established in the last two decades as a crucial transmitter between the nervous and the immune system. DA affects several if not all immune cells, including T and B cells, dendritic cells, macrophages, microglia, neutrophils and NK cells (Basu and Dasgupta, 2000; Sarkar et al., 2010; Levite, 2012; Pinoli et al., 2017). Immune cells themselves may produce and utilize DA as autocrine/paracrine mediator, prominent examples including dopaminergic inhibition of human CD4+CD25^{high} regulatory T lymphocytes (Cosentino et al., 2007), modulation of naive CD4+ T cells differentiation by dendritic cells-derived DA (Nakano et al., 2009), and enhanced T-B cell interaction by follicular helper T cells-derived DA, resulting in more rapid and enhanced germinal center output (Papa et al., 2017). Dopaminergic modulation of the immune response contributes to pathogenesis and therapeutic response in major human disease such as multiple sclerosis (Cosentino and Marino, 2013; Levite et al., 2017), rheumatoid arthritis (Capellino et al., 2010; Nakano et al., 2011), and possibly also Parkinson's disease (González et al., 2013; Kustrimovic et al., 2016).

DA exerts its effects through receptors belonging to the 7-transmembrane, G-protein coupled receptors, grouped into two families according to pharmacological profile and main second messenger coupling: the D_1-like (D_1 and D_5) and the D_2-like (D_2, D_3 and D_4; Gainetdinov et al., 2017). Both D_1-like and D_2-like dopaminergic receptors (DR) are expressed on human immune cells (McKenna et al., 2002; Kustrimovic et al., 2014), and their molecular and pharmacological heterogeneity represents an opportunity for targeted immunomodulating strategies. For instance, D_1-like DR are involved in the suppression of human CD4+CD25^{high} T lymphocytes regulatory function (Cosentino et al., 2007), dendritic cells-induced differentiation of naive CD4+ T cells towards the Th2 lineage (Nakano et al., 2009), and follicular helper T cells-induced B cell maturation (Papa et al., 2017), while D_2-like DR induce T cell quiescence by inhibiting ERK1/ERK2 phosphorylation resulting in up-regulation of Kruppel-like factor-2 expression (Sarkar et al., 2006).

Among human immune cells, CD4+CD25^{high} regulatory T

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lymphocytes, which play a key role in immune homeostasis, express a prominent dopaminergic phenotype and are peculiarly sensitive to the effects of DA. Some of us previously reported that these cells express both D_1-like and D_2-like DR, tyrosine hydroxylase (TH, EC 1.14.16.2, the first and rate-limiting enzyme in the synthesis of catecholamines), and vesicular monoamine transporter (VMAT) 2, which transports DA (and other monoamine transmitters such as noradrenaline, adrenaline, serotonin, and histamine) into storage vesicles (Cosentino et al., 2007). In human CD4 + CD25^{high} regulatory T cells, DA acting on D_1-like DR subserves an autocrine/paracrine inhibitory loop leading to impairment of the regulatory activity exerted by these cells on effector T lymphocytes (Cosentino et al., 2007). Dysregulation of such dopaminergic immune pathway is likely to have clinical relevance, e.g. in multiple sclerosis and its response to immunomodulatory treatment with interferon (IFN)-β (Cosentino et al., 2012).

CD4 + CD25^{high} regulatory T cells originate from thymus and are also termed 'naturally occurring', in contrast to other types of CD4 + T regulatory cells such as Tr1 and Th3, which likely develop from conventional CD4 + CD25- T lymphocytes (Teff) in the periphery (Rutella and Lemoli, 2004; Thompson and Powrie, 2004; Piccirillo and Thornton, 2004). Over the last decade, however, various cell subsets in the CD8 + T cells compartment have been also identified and characterized as suppressors of the immune response (Filaci et al., 2001). CD8 + T regulatory/suppressor cells (CD8 + Treg) affect peripheral tolerance through several mechanisms (Kim and Cantor, 2011), and may be involved in autoimmune diseases (Filaci et al., 2001) as well as in cancer (Filaci et al., 2007; Kiniwa et al., 2007; Wang, 2008; Andersen et al., 2009; Chaput et al., 2009; Parodi et al., 2013).

Circumstantial evidence suggests that DA may affect human CD8 + T cells as well. DA may indeed inhibit the cytotoxicity of CD8 + T cells possibly acting on D_1-like DR (Saha et al., 2001a, 2001b), and may induce migration and homing of naive CD8 + T cells through D_2-like DR D_3 (Watanabe et al., 2006). No information however exists regarding the possible effects of DA on CD8 + Treg. The present study was therefore undertaken to investigate the dopaminergic phenotype of human CD8 + Treg as well as the ability of DA to affect generation and activity of these cells.

2. Materials and methods

2.1. Flow cytometric analysis of DR expression on CD8 + T cells in whole blood

DR expression was assessed on CD8 + T cells and on CD8 + CD28 + and CD8 + CD28- T cells, both in whole blood and in isolated CD8 + T cells before and after 7-day culture. Analysis in whole blood was performed in two steps. In the first step, 100 µl aliquots of whole blood were prepared from each subject (5 were used for DR staining and 1 as control for the secondary PE-goat anti-rabbit (PEGAR) ab), and erythrocytes were removed by means of a lysis buffer ((g/l) NH_4Cl 8.248, $KHCO_3$ 1.0, EDTA 0.0368). Samples were then centrifuged, supernatants were removed and cells were washed in PBS ((g/l) NaCl 8.0, KCl 0.2, Na_2HPO_4 1.42, KH_2PO_4 0.24, pH 7.4) supplemented with 1% BSA (PBS/BSA), and finally resuspended in 45 µl PBS/BSA for staining with one of the five DR by indirect labeling procedure. Briefly, samples were stained with primary ab (final dilution 1:100) and incubated for 30 min on ice. After washing, samples were incubated with PEGAR ab for 30 min on ice in the dark. During the second step, all the aliquots were resuspended in 50 µl PBS/BSA and incubated with a cocktail of anti-human CD3, CD8 and CD28 ab for the identification of CD8 + T lymphocytes and of CD28- and CD28 + cell subsets. After 20 min on ice in the dark, samples were washed and resuspended in 350 µl PBS/BSA and left on ice until acquisition. The gating strategy is shown in Supplementary Fig. S1 online (panel A). Acquisition was then performed on a BD FACSCanto II flow cytometer (Becton Dickinson, Milan, Italy) with BD FACSDiva software (version 6.1.3). Lymphocytes were identified by means of their classical forward scatter (FSC) and side scatter (SSC) signals and a minimum of 20,000 lymphocytes from each sample was collected in the gate. Data were analyzed with the FACSDiva software and the results were finally expressed as percentage of positive cells (%).

For cell culture experiments, CD8 + T cells were isolated as below described. Aliquots containing 3×10^5 purified CD8 + T lymphocytes were first stained for all five DR ab as described above. Cells were then washed and incubated with the appropriate secondary antibody (PE or FITC goat anti-rabbit ab) for 30 min on ice. After washing, cells were incubated with a cocktail of anti-human CD3, CD8, CD28 and CD127 ab, to identify of CD8 + CD28- and CD8 + CD28 + cell subsets and characterize the CD8 + Treg phenotype. After 20 min on ice in the dark, cells were washed and analyzed by a BD FACSCanto flow cytometer (Becton Dickinson, Milan, Italy) with BD FACSDiva software (version 6.1.3). The gating strategy is shown in Supplementary Fig. S1 online (panel B). Cell viability was tested by flow cytometry using the LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit (Thermo Fisher Scientific, Waltham, Massachusetts), with the 405 nm violet laser. Ab used in the study are listed in Supplementary Table S1 online (panel A).

2.2. Isolation of CD8 +, CD8 + CD28- and CD8 + CD28 + T lymphocytes from whole blood

PBMCs were separated from whole blood by Ficoll-Paque Plus (Pharmacia Biotech (Uppsala, Sweden, or Biochrom Ltd., Cambridge, UK) density-gradient centrifugation. Cells were then washed two times in PBS-1 × (pH 7.39). Typical PBMC preparations contained about 82% lymphocytes and 16% monocytes as assessed by flow cytometry. CD8 + T cells were purified using Dynal CD8 positive Isolation kit (Oslo, Norway). CD8 + CD28 + and CD8 + CD28- T cells were subsequently isolated from the CD8 + T cell preparations by sequential cycles of cell sorting on magnetic beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) following the manufacturer's instructions. To this end, CD8 + T cells were labelled with the primary mouse IgG1 antibody (anti-CD28 9.3 mAb - Miltenyi Biotec) and microbeads conjugated with Anti-mouse IgG1 were used for positive selection of CD8 + CD28 + and negative selection of CD8 + CD28- T cells. The purity of CD8 + T cell subsets was tested by 3-color flow cytometric analysis, as above described, and was always ≥ 95%.

2.3. Real time PCR assays of CD8 + T cells

CD8 + T cells, CD8 + CD28 + and CD8 + CD28- cell subsets were isolated as described above, at least 50,000 cells per sample were thereafter resuspended in *PerfectPure RNA lysis buffer* (5 Prime Gmbh, Hamburg, Germany), and total RNA was extracted by *PerfectPure RNA Cell Kit*™ (5 Prime Gmbh). Total RNA was extracted by *PerfectPure*™ *RNA Cell & Tissue kit* (5Prime). The amount of extracted RNA was estimated by spectrophotometry at λ = 260 nm. Total RNA was reverse transcribed using a random primer and a high-capacity cDNA RT kit (Applied Biosystems, Foster City, USA). Amplification of cDNA was then performed by a StepOne® apparatus (Applied Biosystems) using the assays for the genes of interest (BioRad). Real-time PCR assays and conditions are shown in Supplementary Table S1, online (panel B).

Linearity of assays was tested by constructing standard curves using serial 10-fold dilutions of a standard calibrator cDNA for each gene. Regression coefficients (r(Sarkar et al., 2010)) were always > 0.999. Assays were performed in triplicate for each sample, and levels of mRNA were finally expressed as $2^{-\Delta Ct}$ where ΔCt = [Ct (sample) - Ct (housekeeping gene)]. Relative expression was determined by normalization to expression of *RPS18*, which is the gene for 18S cDNA. Data analysis was performed by StepOne software™ 2.2.2 (Applied Biosystems).

2.4. Immunocytochemical analysis of TH and VMAT2

Immunocytochemistry was performed on paraformaldehyde-treated cells flattened over glass slides (0.35×10^6 cells/slide). Cells were rehydrated and treated with 0.1 M glycin in PBS (pH 7.4) followed by 0.3% Triton X-100 buffer, and then incubated overnight at 4 °C with rabbit anti-tyrosine hydroxylase Ab and guinea pig anti-VMAT2 Ab (Immunological Sciences, Rome, Italy). Cells were then washed and stained with rodhamine-conjugated donkey anti-rabbit and 488-conjugated donkey anti-guinea pig antibodies (Invitrogen, Carlsbad, CA) for 45 min at room temperature. TOTO-3 iodide (642/660) (Invitrogen) was used for staining of nuclei. Confocal microscopy was carried out on a Radiance 2100 laser scanning confocal microscope (Biorad Laboratories, Hercules, CA, USA) equipped with a Krypton/Argon laser and a red laser diode with excitation wavelengths set at 488, 546 and 642 nm, respectively.

2.5. High performance liquid chromatography (HPLC) with multi-electrode electrochemical detection

DA (together with the other catecholamines noradrenaline and adrenaline) were assayed by HPLC with multi-electrode electrochemical detection (HPLC-ED), as described previously (Cosentino et al., 2000, 2007). Briefly, 1×10^6 cells were centrifuged for 5 min at $1400 \times g$ and 20 °C, the medium was removed, filtered, added with 3,4-dihydroxybenzylamine hydrobromide as internal standard, and 30 µl were injected into the HPLC system. The cell pellet was resuspended in 0.2 ml of $HClO_4$ 0.1 N and disrupted by sonication. The mixture was then centrifuged for 5 min at 15,000 × *g* and 4 °C and the supernatant was recovered, filtered and 30 µl were injected into the chromatographic system. The medium was added with $HClO_4$ 0,4 N (1:1 *v*/v), kept on ice for 30 min, and then centrifuged for 5 min at 15,000 × *g* and 20 °C. The supernatant was recovered, filtered and 30 µl were injected into the HPLC system. The chromatograms were collected, stored and processed with the application software Coularray for Windows (ESA). CA in the samples were quantified by using the peak heights of a standard curve generated by injecting known samples (3 fmol–3 pmol), and values were finally normalized for protein content, assessed by means of the bicinchoninic acid (BCA) protein assay (Smith et al., 1985).

2.6. Generation of CD8+ Treg

Peripheral blood mononuclear cells (PBMC) were separated from buffy coats by density gradient centrifugation using Ficoll-Hypaque (Biochrom AG, Berlin) for 30 min at 1800 rpm. CD8+ T cells were isolated by cell sorting on magnetic beads using microbeads conjugated with mAb specific for the CD8 antigen (Dynal CD8 positive isolation kit, Invitrogen by Life Technologies Ltd., Paisley, UK), according to the manufacturer's instructions. The purity of CD8+ T cells was always ≥95% as assessed by flow cytometric analysis.

Purified CD8+ T lymphocytes (1×10^6 cells/ml, 2×10^5 cells/well) were incubated in 96-well flat bottomed plate in the presence of RPMI-1640 culture medium (Gibco by Life Technologies Ltd., Paisley, UK) supplemented with 10% FCS (Invitrogen by Life Technologies Ltd., Paisley, UK), 20 U/ml IL-2 (Proleukin, Eurocetus, Amsterdam, The Netherlands) and 10 ng/ml of IL-10 (PeproTech, Rocky Hill, NJ, USA) at 37 °C for 7 days. At the end of the incubation, the cells were collected, washed, counted, and used as suppressors in the proliferation suppression assay.

2.7. Proliferation suppression assay

The suppressive activity of CD8+ Treg was evaluated by monitoring the dilution of a carboxyfluorescein succinimidyl ester (CFSE) dye (Molecular Probes, Invitrogen) in human PBMC by means of flow cytometry. PBMC from healthy donors were stained with CSFE (5 µM) and pulsed with mouse anti-human CD3 mAb (5 µg/ml, BD Biosciences, San Josè, CA) and mouse anti-human CD28 mAb (5 µg/ml, BD Biosciences, San Josè, CA) for 3 h. Then cells were washed and cultured for 5 days in a 96-well flat-bottomed plate (1×10^5 cells/well) either alone or in the presence of CD8+ Treg (1×10^5 cells/well), taken before and after in vitro generation. At the end of the incubation, samples were washed in PBS and analyzed using a FACSCanto flow cytometer (BD Bioscience, Franklin Lakes, NJ) using FACSDiva software (BD Biosciences, Franklin Lakes, NJ). Dead cells were excluded from the analysis by adding 7-aminoactinomycin D (BD Biosciences) before the acquisition.

2.8. Statistics

Data are presented as means ± SEM, unless otherwise indicated. Statistical significance of the differences between groups was assessed by two-tailed Student's *t*-test or one-way analysis of variance followed by Bonferroni or Dunnet post-test for paired or unpaired data, as appropriate. Calculations were performed using a commercial software (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com).

3. Results

3.1. CD8+ T cells express DR, TH and VMAT2 and contain high levels of intracellular dopamine

In whole blood, CD8+ T cells were 8.1 ± 0.5% of total lymphocytes ($n = 3$), and CD8+CD28+ and CD8+CD28$^-$ cells were respectively 66.7 ± 8.5% and 34.6 ± 8.7% of CD8+ T cells. CD8+ T cells expressed all the five DR, which were present on average on 6.0–12.9% of the cells with no differences between CD8+CD28+ and CD8+CD28$^-$ cells (Fig. 1). CD8+ T cells as well as CD8+CD28+ and CD8+CD28$^-$ T cell subsets also expressed mRNA for all the five DR, although to a different extent. Indeed, mRNA levels for both D_1-like DR D_1 and D_5 as well as for D_2-like DR D_2, D_3 and D_4 were higher in CD8+CD28- T lymphocytes in comparison to CD8+CD28+ T cells (Fig. 2). CD8+ T cells also contained mRNA for TH, the first and rate-limiting enzyme in the synthesis of dopamine, and for VMAT2 (but not for VMAT1). VMAT2 mRNA was higher in CD8+CD28$^-$ than in CD8+CD28+ T cells, while TH mRNA was not different between the subsets (Fig. 2). The presence of TH and VMAT2 in both CD8+CD28+ and CD8+CD28$^-$ T cells was confirmed also by means of immunocytochemical analysis, which showed their localization in the thin cytoplasmic layers surrounding cell nuclei (Fig. 3). DA levels were higher in CD8+CD28$^-$ T cells in comparison to CD8+CD28+ T cells (23.8 ± 6.1 vs 1.6 ± 0.3 pmol/mg of proteins, corresponding to 1.36 ± 0.32 vs 0.10 ± 0.02 pmol/10^6 cells, $n = 5$, $P = 0.023$) (Supplementary Fig. S2 online, panel A). Compared to CD8+CD28+ T cells, CD8+CD28 CD8+CD28$^-$ T lymphocytes contained also higher levels of noradrenaline and adrenaline (Supplementary Fig. S2 online, panel B).

3.2. Generation of CD8+ Treg is associated with preferential upregulation of DR mRNA levels in the CD8+ CD28- T cell compartment

Non-antigen specific CD8+ Treg can be generated in vitro through incubation of CD8+ T cells with IL-2 and IL-10 for 7 days. CD8+ Treg derive from CD8+CD28$^-$ T cell precursors and belong themselves to the CD8+CD28- T cell compartment(Filaci et al., 2004a, 2004b). In the present study, we investigated DR expression on CD8+ T cells before and after generation of CD8+ Treg. To this end, CD8+ Treg were generated in vitro from PBMC of 4 healthy donors. Preliminarily, freshly purified CD8+ T cells were tested and no suppressive activity was detected (not shown), in agreement with previous data by some of

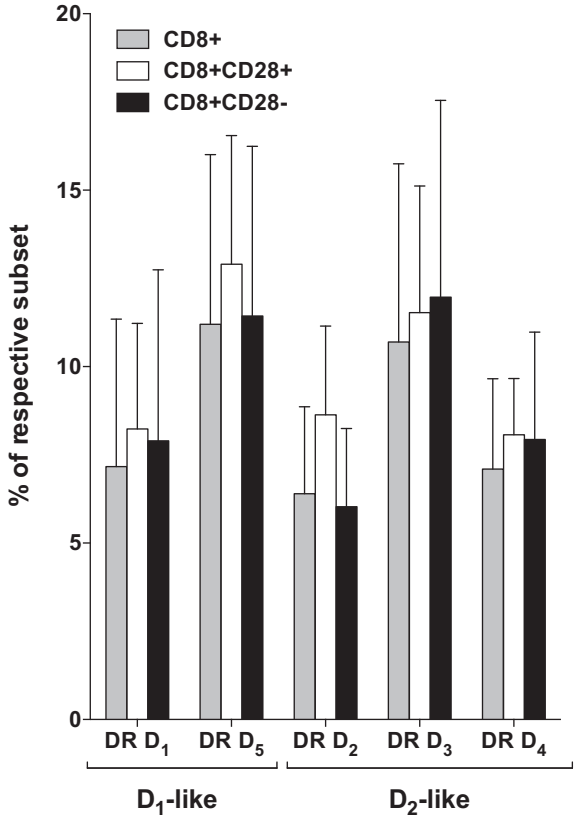

Fig. 1. Expression of DR on circulating CD8+ T cells.
The frequency of DR+ cells is shown as are means ± SEM percentage of CD8+ (shaded), CD8+CD28+ (empty), and CD8+CD28- cells (filled).

us showing that no remarkable (> 25%) suppressor activity is mediated by circulating CD8+ T cells in healthy subjects (Filaci et al., 2001, 2004). After 1-week incubation with IL-2 and IL-10, generation of CD8+ Treg was confirmed by phenotypic and functional analyses. CD8+ Treg showed CD127 downregulation and CD39 expression, both typical of the CD8+ regulatory T cell phenotype (Liu et al., 2006; Kustrimovic et al., 2018) (Supplementary Fig. S1 online, panel B) and acquisition of effective suppressive function (Supplementary Fig. S3 online).

DR expression was analyzed on both CD8+CD28+ and CD8+CD28$^-$ T cell subsets before and after in vitro generation of CD8+ Treg. In comparison to CD8+CD28+ and CD8+CD28$^-$ T cells contained in freshly isolated CD8+ T cells, both fractions after CD8+ Treg generation showed increased expression of the D_2-like DR D_4 on the cell surface (Fig. 4) and increased mRNA levels for the D_1-like DR D_5 (Fig. 5), however only the CD8+CD28- T cell fraction showed increased mRNA levels for the D_1-like DR D_1 and for the D_2-like DR D_3 and D_4 (Fig. 5). In CD8+CD28+ T cells the T7/T0 ratio of mRNA levels for the D_1-like DR D_5 was 1.98 ± 0.19, while in CD8+CD28- T cells the T7/T0 ratio of mRNA levels for the D_1-like DR D_1 and D_5 were respectively 2.61 ± 0.49 and 2.70 ± 0.34, for the D_2-like DR D_3 and D_4 were 1.55 ± 0.10 and 2.10 ± 0.39.

3.3. Dopamine affects both generation and suppressive activity of CD8+ Teg

In a first set of experiments, the suppressive activity of CD8+ Treg generated under standard conditions was compared with that of CD8+ Treg generated in the presence of DA added at the beginning of cell culture. The presence of DA alone or together with the D_1-like DR antagonist SCH-23390 did not affect to any extent the subsequent ability of CD8+ Treg to suppress PBMC proliferation. Generation of functional CD8+ Treg was on the contrary prevented by DA added together with the D_2-like DR antagonist haloperidol (Fig. 6, panel A), as well as by incubation of the cells with the D_1-like DR agonist SKF-38393 (Fig. 6, panel B).

The effect of DA was also tested by adding DA at the beginning of CD8+ Treg-PBMC coincubation in the proliferation suppression assay. The presence of DA reduced the suppressive activity of CD8+ Treg, and the effect was mimicked by the D_1-like DR agonist SKF-38393 (Fig. 6).

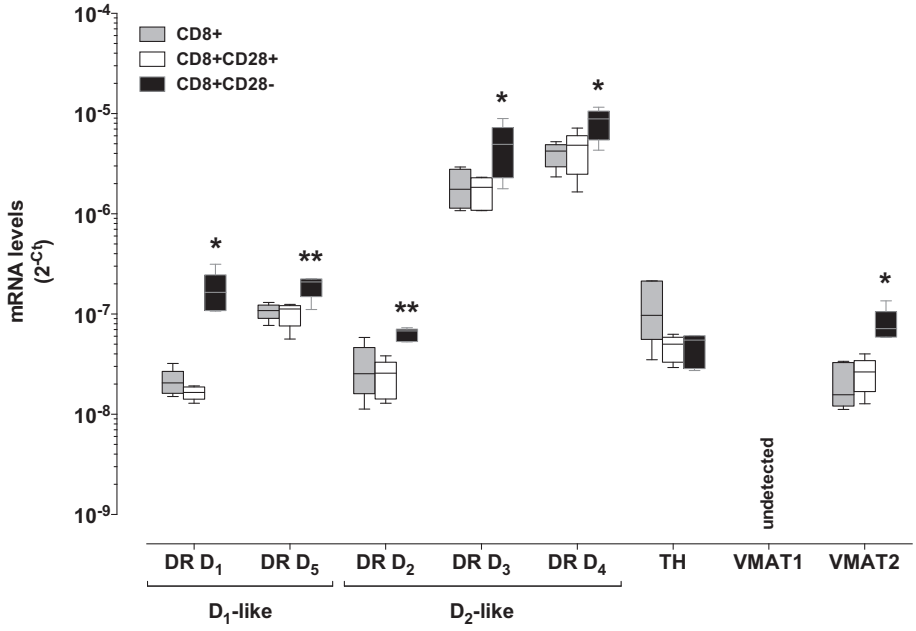

Fig. 2. DR, TH and VMAT mRNA levels in CD8+ T cells.
Data are medians with 25th–75th percentiles (boxes) and min-max values (whiskers) of mRNA levels in CD8+ (shaded), CD8+CD28+ (empty), and CD8+CD28- cells (filled). * = $P < .05$ and ** = $P < .01$ vs CD8+CD28+ cells.

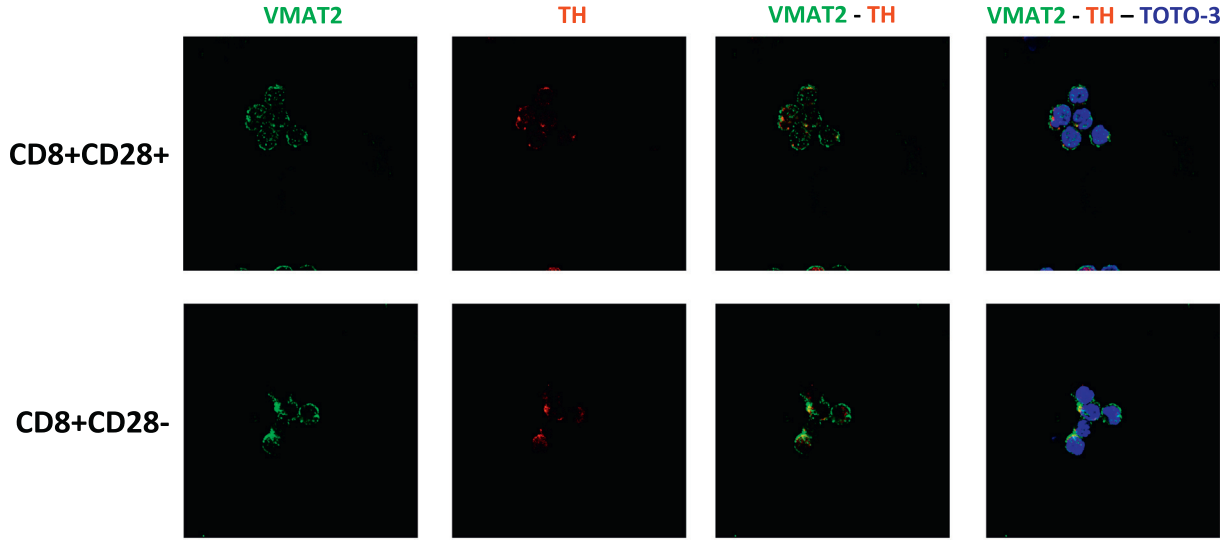

Fig. 3. Immunostaining of VMAT2 and TH in CD8+ T cell subsets.
Paraformaldehyde-fixed cells were flattened over glass slides and stained for VMAT2 (green) and TH (red), and treated with TOTO-3 (blue) to color nuclei. VMAT2 and TH immunoreactivity is present in both CD8+CD28+ and CD8+CD28- T cells and is localized in the thin cytoplasmic layer surrounding cell nuclei. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

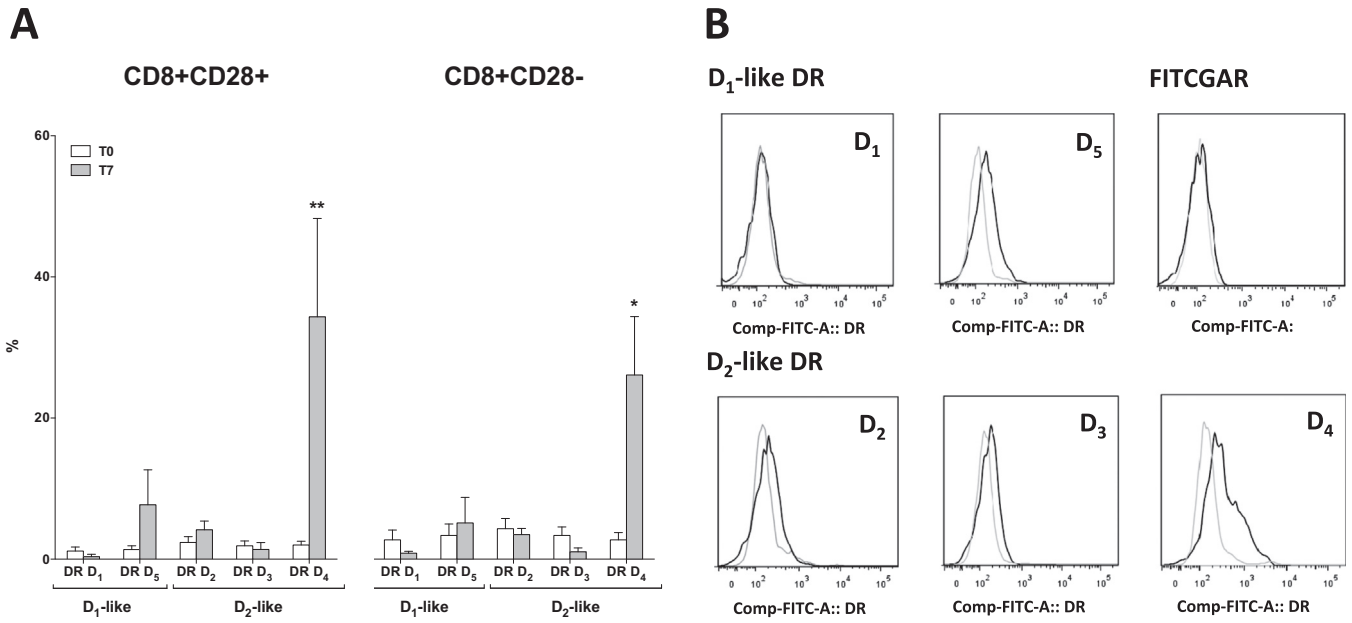

Fig. 4. Flow cytometric analysis of DR expression on CD8+CD28+ and CD8+CD28- T cells before and after CD8+ Treg generation.
Panel A: the frequency of DR+ cells is shown as means ± SEM percentage of total CD8+CD28+ and CD8+CD28- T cells at T0 (empty) and T7 (shaded). * = $P < .05$ and ** = $P < .01$ vs T0. Panel B: DR expression on CD8+CD28- T cells from a representative subject. In each histogram, grey lines are T0 and black lines are T7. FITCGAR = negative control stained with secondary FITCGAR ab alone.

4. Discussion

The present study provides comprehensive evidence about a key role of dopaminergic pathways in the generation and regulation of human CD8+ Treg. Results can be summarized as follows:

(1) human circulating CD8+ T lymphocytes express both D_1-like and D_2-like DR on the cell surface, to the same extent in the CD8+CD28+ and in the CD8+CD28- fractions, however DR mRNA levels are higher in CD8+CD28- in comparison to CD8+CD28+ T cells;
(2) generation of CD8+ Treg occurs together with increased mRNA levels of D_1-like DR D_1 and D_5 and D_2-like DR D_3 and D_4;
(3) activation of D_1-like DR-operated pathways impairs CD8+ Treg generation and inhibits CD8+ Treg suppressive activity.

Although all D_1-like and D_2-like DR are expressed on the cell surface of CD8+ T cells, they occur in a limited fraction of total cells, on average between 6% and 12.5%. This finding is in line with observations in CD4+ T cells showing that DR+ cells are just 5–12% of total cells (Kustrimovic et al., 2014). Nonetheless, among CD4+ T cells expression may vary according to the specific lineage, as Th1 and Th2 contain less DR+ cells in comparison to Th17 (Kustrimovic et al., 2018). We previously reported that CD4+ Treg are highly sensitive to

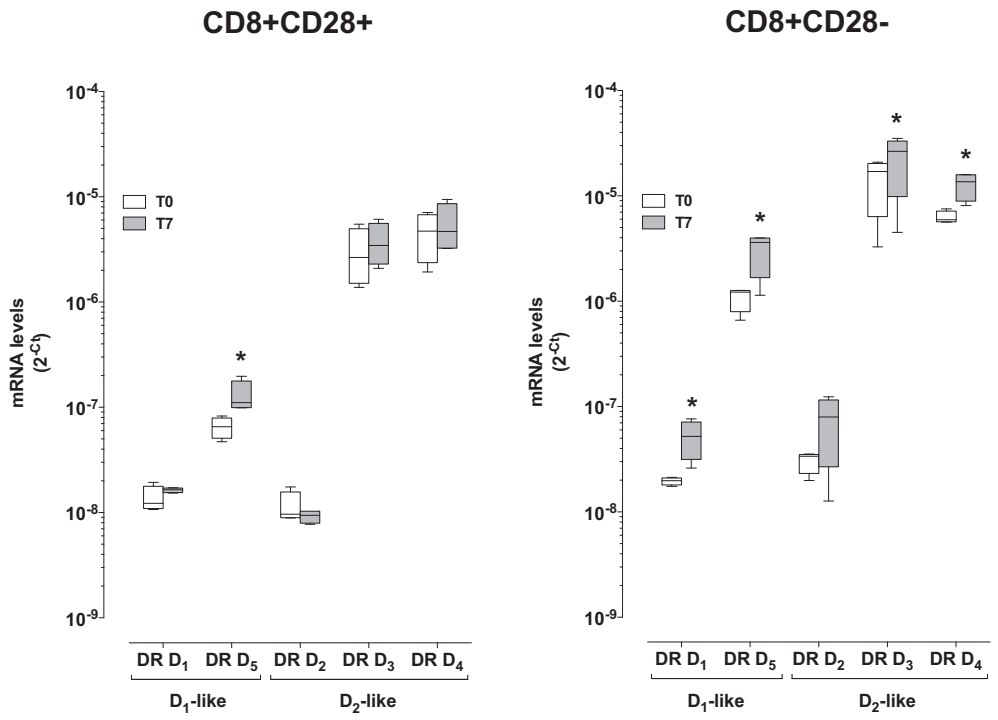

Fig. 5. DR, TH and VMAT mRNA levels in CD8 + 28 + and CD8 + CD28- T cells before and after CD8 + Treg generation.
Data are medians with 25th–75th percentiles (boxes) and min-max values (whiskers) of mRNA levels in cells at T0 (empty) and at T7 (shaded) from 4 separate subjects. * = $P < .05$ vs T0.

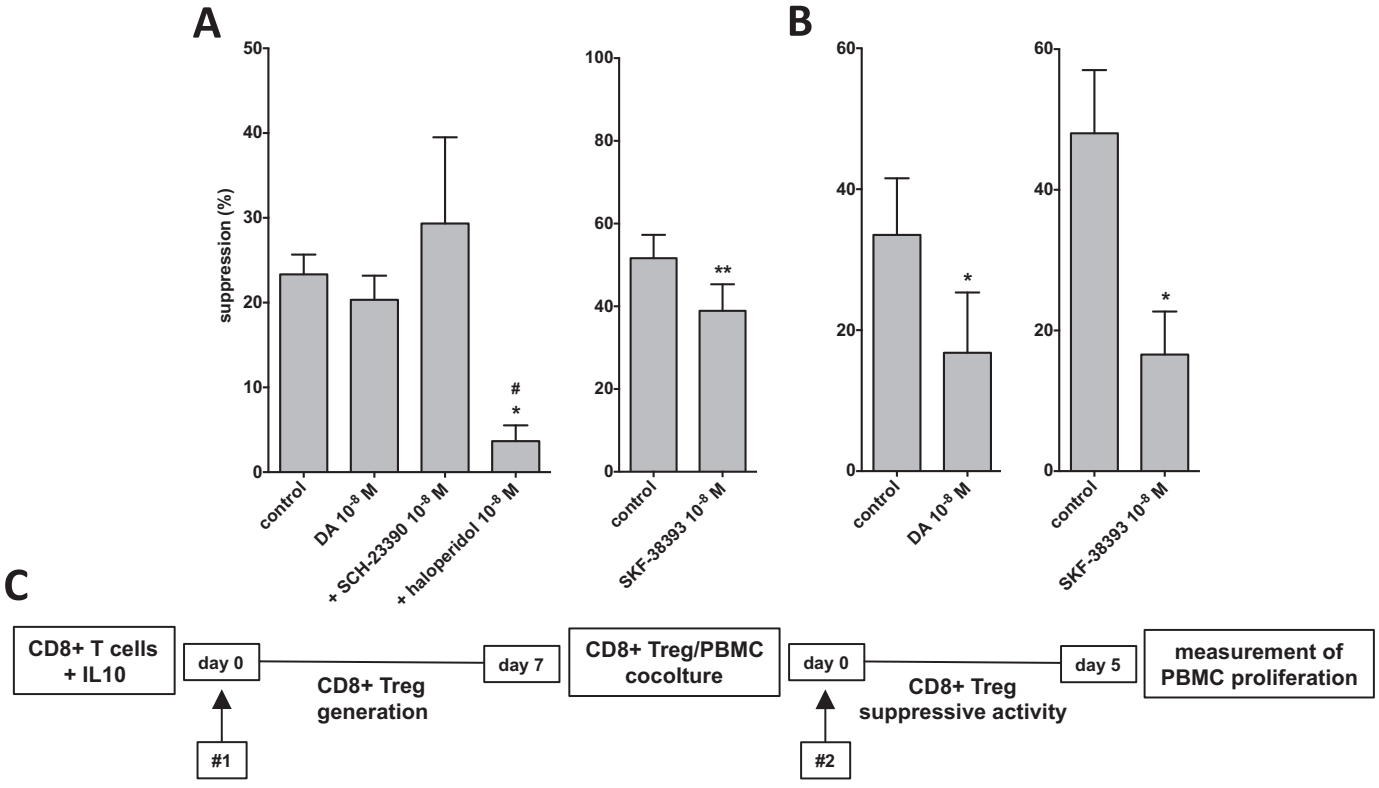

Fig. 6. Effect of DA and of dopaminergic agents on CD8 + Treg generation.
Panel A: effect of DA alone and in the presence of the D_1-like DR antagonist SCH-23390 or the D_2-like DR antagonist haloperidol. Panel B: effect of the D1-like DR agonist SKF-38393. Data are means ± SEM. * = $P < .05$ and ** = $P < .01$ vs respective control, and # = $P < .05$ vs DA.

dopamine, which effectively suppresses their regulatory functions (Cosentino et al., 2007; Kustrimovic et al., 2018), nonetheless among CD4+ Treg DR+ cells are on average 12–15% for all D_1-like and D_2-like DR (Kustrimovic et al., 2018), possibly suggesting that the frequency of DR+ cells is not critical for the ability of T cells to respond to dopamine. It might be that DR are preferentially expressed on most active cells and/or that DR+ cells in response to dopamine produce mediators which in turn affect also DR- cells. It must be also considered that current assays of DR on immune cells allow the identification of one DR at a time, due to the availability of antibodies all obtained in rabbit and pertaining the to same IgG class (Supplementary Table S1). It cannot be excluded that the five DR are expressed on different fractions of the same cell population, resulting in higher percentages of DR+ cells defined as expressing any subtype of DR. To clarify the issue, primary anti-DR antibodies conjugated with different fluorochromes should be developed for flow cytometry applications, thus allowing the simultaneous detection of different DR on the same cells.

Results also show that CD8+ T cells express TH, the rate-limiting enzyme in the synthesis of dopamine as well as of noradrenaline and adrenaline, and VMAT2, which is responsible for monoamine storage in secretory vesicles and granules. Presence of TH and VMAT2 has been documented at the level of both mRNA ad protein. Remarkably, immunocytochemistry showed that TH and VMAT2 are located in the thin cytoplasmic layer surrounding cell nuclei. We previously reported that in human PBMC TH-specific immunoreactivity is localized to the plasma membrane and to electron-dense cytoplasmic granules, which resemble those found in neurosecretory PC12 cells (Reguzzoni et al., 2002). As for VMAT2, its presence was documented at the mRNA level and by means pharmacological tools in CD4+ Treg (Cosentino et al., 2007), which similar to CD8+ T cells do not express mRNA for VMAT1. In agreement with the presence of TH and VMAT2, CD8+ T cells also contain significant amounts of dopamine, noradrenaline and adrenaline, which are higher in the CD8+CD28- in comparison to the CD8+CD28+ cell subset. Anyway, catecholamine levels in CD8+CD28- T cells although quite high are nevertheless lower than those reported in CD4+ Treg cells (on average, dopamine: 37.5 pmol/10^6 cells in CD4+ Treg vs 1.4 pmol/10^6 cells in CD8+CD28- T cells; noradrenaline: 26.6 vs 5.53; adrenaline: 25.2 vs 2.8; Cosentino et al., 2007). It remains therefore to be established whether such amounts may be enough to subserve any autocrine/paracrine functional loop in CD8+28- T cells similar to that occurring in CD4+ Treg, however the present findings significantly extend previous observations regarding the occurrence of a whole dopaminergic/catecholaminergic molecular machinery in human T cells.

The involvement of dopaminergic pathways in the generation and function of CD8+ Treg is supported by both molecular and pharmacological evidences. Indeed, generation of CD8+ Treg occurs together with increased expression of mRNA levels for all the D_1-like DR and D_2-like DR (with the only exception of the D_2-like DR D_2) and is impaired by activation of D_1-like DR-operated pathways. Interestingly, dopamine alone was unable to modify the generation of CD8+ Treg, but resulted in effective inhibition in the presence of the D_2-like DR antagonist haloperidol, while the D_1-like DR antagonist SCH-23390 did not exert any effect. Dopamine acts on both D_1-like and D_2-like DR, therefore the effect observed in the presence of haloperidol (i.e. under experimental conditions which likely prevent dopamine from binding to D_2-like DR) likely depend on the selective stimulation of D_1-like DR alone. In agreement with such hypothesis, generation of CD8+ Treg in the presence of the D_1-like DR agonist SKF-38393 resulted in reduced suppressive activity by CD8+ Treg, thus leading to the conclusion that CD8+ Treg generation can be inhibited by activation of D_1-like DR. Activation of D_1-like DR-operated pathways nonetheless also resulted in the impairment of CD8+ Treg suppressive activity, as suggested by the enhanced proliferation of PBMC in the presence of dopamine or SKF-38393 in the CD8+ Treg-PBMC proliferation suppression assay.

The present results support a key role of D_1-like DR-operated pathways in human T cells as negative modulators of regulatory functions also in the CD8+ compartment, similar to what some of us previously reported in CD4+ T cells (Cosentino et al., 2007, 2012). D_1-like DR-dependent inhibition of CD4+ and CD8+ Treg function opens up the opportunity to examine D_1-like DR-selective agonist as potential immunoenhancing drugs. Interestingly, in human T cells D_1-like DR activation may induce β1 integrin-dependent adhesion to fibronectin (Levite et al., 2001) as well as increased production of TNF-α (Besser et al., 2005). Nonetheless, circumstantial evidence suggests that under some circumstances D_1-like DR activation may also result in inhibition of cytotoxic activity in CD4+ and CD8+ T cells (Saha et al., 2001a, 2001b).

Nearly four decades ago dopamine was suggested as a novel antitumor agent against e.g. melanoma (Wick, 1982), however its possible mechanism(s) of action remained ill-defined (FitzGerald and Wick, 1983). Improved efficacy and reduced hematotoxicity of cyclophosphamide were described in a mouse model of carcinoma after treatment with DA (Lakshmi et al., 2005). DA has been reported to act as an endogenous inhibitor of tumor angiogenesis, possibly by down-regulating VEGFR-2 signaling pathways in endothelial cells (Basu et al., 2001, 2004). In vivo DA inhibits tumor angiogenesis and increases the survival of animals transplanted with human malignant tumors (Chakroborty et al., 2004, 2008). In view of the key role of Treg as a crucial strategy of tumors to escape the host immune system (Zitvogel et al., 2006; Nishikawa and Sakaguchi, 2014; Bhatia and Kumar, 2014), the ability of dopamine to inhibit the regulatory function of both CD4+ and CD8+ Treg adds to its potential as an antitumor agent, and strengthens the notion that dopaminergic pathways could represent druggable targets to develop original and innovative antitumor therapeutic strategies.

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Competing financial interests

The authors declare that they have no competing financial interests.

Author contribution

Study conception and design

DF, FM, GF and MC.

Acquisition of data

GN, TA, ER, AP, FK, DF, FM.

Analysis and interpretation of data

GN, TA, DF, FM, GF and MC.

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved, and declare to have confidence in the integrity of the contributions of their co-authors.

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