


# cAMP levels in lymphocytes and CD4<sup>+</sup> regulatory T-cell functions are affected by dopamine receptor gene polymorphisms

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## Summary

The neurotransmitter dopamine (DA) has prominent effects in the immune system and between the immune cells, CD4<sup>+</sup> regulatory T (Treg) lymphocytes, a specialized T-cell subset crucial for the control of immune homeostasis, are especially sensitive to DA. Dopaminergic receptors (DR) are grouped into two families according to their pharmacological profile and main second messenger coupling: the D<sub>1</sub>-like (D<sub>1</sub> and D<sub>5</sub>), which activate adenylate cyclase, and the D<sub>2</sub>-like (D<sub>2</sub>, D<sub>3</sub> and D<sub>4</sub>), which inhibit adenylate cyclase and exist in several variants that have been associated to clinical conditions such as schizophrenia, bipolar disorder, substance abuse and addiction. We aimed to examine, in venous blood samples from healthy volunteers, the relationship between the arbitrary DR score and DR functional responses in human lymphocytes. All the samples were genotyped for selected DR gene variants (*DRD1*: rs4532 and rs686; *DRD2*: rs1800497 and rs6277; *DRD3*: rs6280; *DRD4*: rs747302 and seven 48-base pair variable number tandem repeat (VNTR)) and a DR score was attributed to each participant. We have also tested whether DR gene polymorphisms might affect Treg cell ability to suppress effector T-cell function. To our knowledge, this is the first study showing a correlation between DR gene variants and human T lymphocyte function. The main results are that both D<sub>1</sub>-like and D<sub>2</sub>-like DR are functionally active in human lymphocytes, although the D<sub>1</sub>-like DR stimulation results in stronger effects in comparison to the D<sub>2</sub>-like DR stimulation. In addition, it seems that the DR genetic profile may affect the ability of lymphocytes to respond to dopaminergic agents. More investigations are needed about the possible clinical relevance of such findings.

**Keywords:** CD4 cell; immune homeostasis; inflammation; regulatory T cell.

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The neurotransmitter dopamine (DA) has prominent effects in the immune system, affecting T and B lymphocytes, dendritic cells, monocytes/macrophages, neutrophils and natural killer cells.<sup>1,2</sup> Among lymphocytes, CD4<sup>+</sup> regulatory T (Treg) lymphocytes, a specialized T-cell subset crucial for the control of immune homeostasis, are especially sensitive to DA, which down-regulates their regulatory functions acting on D<sub>1</sub>-like dopaminergic receptors (DR).<sup>3</sup>

Dopaminergic receptors belong to the seven-transmembrane, G-protein coupled receptors family, and are grouped into two families according to their pharmacological profile and main second messenger coupling: the D<sub>1</sub>-like (D<sub>1</sub> and

D<sub>5</sub>), which activate adenylate cyclase, and the D<sub>2</sub>-like (D<sub>2</sub>, D<sub>3</sub> and D<sub>4</sub>), which inhibit adenylate cyclase.<sup>4</sup> DR genes exist in several variants, which have been associated with clinical conditions such as schizophrenia, bipolar disorder, substance abuse and addiction.<sup>5,6</sup>

We recently showed that in the peripheral blood of healthy individuals CD4<sup>+</sup> T lymphocyte count is associated with DR gene polymorphisms, and in particular with an arbitrary score based on the activity of D<sub>1</sub>-like versus D<sub>2</sub>-like DR (higher values corresponding to increased D<sub>1</sub>-like DR density/responsiveness), suggesting that increased D<sub>1</sub>-like DR activity might correlate with a higher number of circulating CD4<sup>+</sup> T cells.<sup>6</sup>

The aim of the present investigation was first to examine the relationship between the DR score used in the previous study and DR functional responses in human lymphocytes. To this end, we collected venous blood samples from healthy volunteers, as part of a project aimed at assessing the expression and role of DR on circulating CD4<sup>+</sup> T cells in Parkinson's disease. The study protocol was approved by the Ethics Committee of the Ospedale di Circolo and Fondazione Macchi, Varese (I).<sup>7</sup> Lymphocytes were isolated by Ficoll-Paque Plus density gradient centrifugation, resuspended in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum and 2 mM glutamine, at a final concentration of  $1 \times 10^6$  cells/ml for subsequent culture at 37° in a moist atmosphere of 5% CO<sub>2</sub>. Cells were then stimulated for 45 min with phytohaemagglutinin (PHA) 10 µg/ml, alone or in the presence of the D<sub>1</sub>-like DR agonist SKF-38393, 1 µM, or the D<sub>2</sub>-like DR agonist pramipexole, 1 µM. Cells were finally harvested and intracellular cAMP was measured using a cAMP ELISA kit (R&D Systems, McKinley Place, NE, Minneapolis, MN). All the samples were genotyped for selected DR gene variants (*DRD1*: rs4532 and rs686; *DRD2*: rs1800497 and rs6277; *DRD3*: rs6280; *DRD4*: rs747302 and seven 48-base pair variable number tandem repeat (VNTR)) and a DR score was attributed to each participant, as previously described.<sup>6</sup> Briefly, +1 was assigned to each allele associated with increased D<sub>1</sub>-like DR activity or decreased D<sub>2</sub>-like DR activity, and -1 to each allele associated with decreased D<sub>1</sub>-like DR activity or increased D<sub>2</sub>-like DR activity (Table 1).<sup>5,6</sup> In our previous study,<sup>6</sup> this score correlated with total lymphocytes, CD3<sup>+</sup> and CD4<sup>+</sup> T cells in the peripheral blood of healthy individuals.

As expected, stimulation with PHA increased intracellular cAMP by (mean ± SEM)  $65.3 \pm 1.6 \times 10^{-12}$  mol/10<sup>6</sup> cells ( $n = 12$ ,  $P < 0.001$  versus resting cells). In comparison to PHA alone, the increase was higher in the presence of the D<sub>1</sub>-like DR agonist SKF-38393 ( $99.1 \pm 2.3 \times 10^{-12}$  mol/10<sup>6</sup> cells,  $n = 12$ ,  $P < 0.001$  versus PHA alone), and was lower with the D<sub>2</sub>-like DR agonist pramipexole ( $56.4 \pm 1.9 \times 10^{-12}$  mol/10<sup>6</sup> cells,  $n = 12$ ,  $P < 0.001$  versus PHA alone) (Fig. 1a). In individual preparations, the SKF-38393-induced increase of cAMP correlated with the DR score: in particular, the SKF-38393-induced cAMP increase was higher with increasing DR score, corresponding to increased D<sub>1</sub>-like DR density and/or activity, whereas the pramipexole-induced decrease showed no statistically significant correlation with the DR score (Fig. 1b).

In a second set of experiments, we tested whether DR gene polymorphisms might affect the ability of Treg cells to suppress T effector (Teff) cell function. We used venous blood samples collected as described above, and Treg cells were separated from CD4<sup>+</sup> Teff cells by means

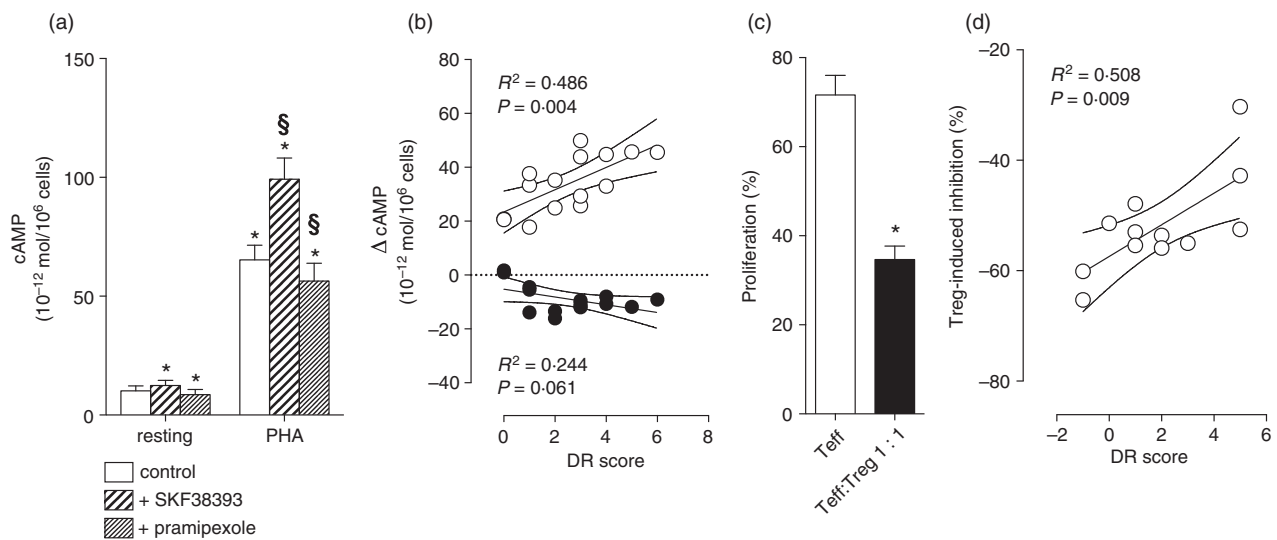
of a human CD4<sup>+</sup> CD25<sup>+</sup> Regulatory T cell Isolation Kit (Miltenyi Biotec, Auburn, CA, USA) as previously reported.<sup>3,8</sup> Treg and Teff cells were then placed in wells containing RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine and 100 U/ml penicillin/streptomycin, at 37° in a moist atmosphere of 5% CO<sub>2</sub>. Cells were kept alone or in co-culture (Teff : Treg ratio = 1 : 1), and treated with PHA 5 µg/ml, and interleukin-2 40 ng/ml. Proliferation of Teff cells was measured after 5 days of culture by using flow cytometry and Cell Proliferation Dye eFluor. Acquisition was performed on a BD FACSCanto II flow cytometer (Becton Dickinson Italy, Milan, Italy) with BD FACSDIVA software (version 6.1.3). Even in this set of experiments, all the samples were genotyped for the previously listed DR gene variants and a DR score was attributed to each participant.

When cultured alone, Teff proliferated up to  $71.6\% \pm 4.4\%$  (mean ± SEM,  $n = 12$ ). Proliferation in the presence of Treg decreased to  $34.7\% \pm 3.0\%$  ( $n = 12$ ,  $P < 0.0001$ ) (Fig. 1c). In individual preparations, Treg-induced reduction of Teff proliferation was inversely proportional to the DR score (Fig. 1d), implying that Treg cell inhibition of Teff cell proliferation decreased with increased D<sub>1</sub>-like DR activity. Analysis of individual DR gene variants showed that the *DRD1* rs4532 was strongly associated with Treg cell inhibition of Teff cell proliferation (Table 2). Co-incubation with DA 1 µM reduced Treg-induced inhibition of Teff cell proliferation by  $63.0\% \pm 10.1\%$  ( $P = 0.001$  versus Treg alone); however, in the two individuals carrying the *DRD1* rs4532 A/A genotype, dopaminergic reduction of the effects of Treg cells was only  $45.7\% \pm 18.1\%$ , whereas in the eight participants with the A/G phenotype it was  $69.0\% \pm 16.8\%$  and in the two participants with the A/A phenotype it was  $65.6\% \pm 12.6\%$ .

This is the first study showing a correlation between DR gene variants and human T lymphocyte function. In our previous investigation,<sup>6</sup> we reported an association between DR gene variants and circulating CD4<sup>+</sup> T-cell count, suggesting that the prevalence of D<sub>1</sub>-like DR activity might lead to increased CD4<sup>+</sup> T cells in peripheral blood. Interestingly, we recently reported that D<sub>1</sub>-like DR are more expressed than D<sub>2</sub>-like DR in human CD4<sup>+</sup> T cells.<sup>7</sup> DR gene variants have been studied so far mainly in relationship to substance addiction and to behavioural responses to stimulants,<sup>5,6</sup> and no information is currently available regarding their role in other diseases and/or in the regulation of immune cell function, despite extensive knowledge about dopaminergic regulation of adaptive and innate immunity in health and disease.<sup>2,9,10</sup> In the present study, we tested the effects of DR gene variants on the short burst of cAMP production induced by PHA stimulation, which is critical for T-cell activation,<sup>11</sup> and on Treg-induced inhibition of Teff cell

**Table 1.** Dopaminergic receptor (DR) gene variants analysed in the study and assigned scores (for detailed references see ref. 6)

Receptor	Gene	Variant	Nucleotide change	Effects	Score
D <sub>1</sub> -like	DRD1	rs4532	-48A>G	Association with nicotine and alcohol dependence, and with tobacco smoking in schizophrenia.	+1
		rs686	62C>T	Higher gene expression and association with nicotine and alcohol dependence, and with tobacco smoking in schizophrenia.	+1
D <sub>2</sub> -like	DRD2	rs1800497	2137G>A	Lower striatal DR D <sub>2</sub> density in healthy individuals.	+1
		rs6277	957C>T	Decreased mRNA stability and translation, reduced dopamine-induced up-regulation of DR D <sub>2</sub> expression <i>in vitro</i> , and lower DR D <sub>2</sub> expression in cortex and thalamus of healthy individuals.	+1
D <sub>3</sub>	DRD3	rs6280	25G>A	Higher dopamine binding affinity <i>in vitro</i> , association with alcohol and heroin dependence.	-1
D <sub>4</sub>	DRD4		7 48-base pair VNTR	Reduced mRNA levels in human post-mortem brain tissue samples, lower response to stimulants and requirements of higher doses of methylphenidate.	+1



**Figure 1.** Dopaminergic receptor (DR) gene polymorphisms affect cAMP responses in peripheral blood mononuclear cells (PBMC) and regulatory T (Treg) cell-induced inhibition of effector T (Teff) cell proliferation. (a) Stimulation with phytohaemagglutinin (PHA) increases cAMP levels in PBMC and the effect is enhanced by the D<sub>1</sub>-like DR agonist SKF-38393 1 μM and reduced by the D<sub>2</sub>-like DR agonist pramipexole 1 μM. Data are means ± SEM of 15 different participants. \**P* < 0.05 versus resting/control and §*P* < 0.05 versus PHA alone. (b) Correlation between SKF-38393-induced increase (empty circles) or pramipexole-induced decrease of cAMP levels in PBMC and DR score. Linear regression lines are represented together with their 95% confidence intervals. *R*<sup>2</sup> and *P* values are indicated in the graph. The effect of SKF-38393 increases with increasing DR score, corresponding to increased D<sub>1</sub>-like DR activity, whereas the effect of pramipexole shows no significant correlation with the score. (c) Proliferation of Teff cells stimulated with PHA/interleukin-2 alone and in the presence of Treg cells 1 : 1. Data are means ± SEM of 12 different participants. \**P* < 0.0001 versus Teff alone. (d) Correlation between Treg-induced inhibition of Teff cell proliferation and DR score. Linear regression lines are represented together with their 95% confidence intervals. *R*<sup>2</sup> and *P* values are indicated in the graph. Treg cell inhibition of Teff cell proliferation decreased with increased DR score and therefore with increased D<sub>1</sub>-like DR activity.

proliferation. Indeed, we previously reported that Treg cells, in comparison to Teff cells, respond to DA with a rapid and sustained increase of cAMP.<sup>3</sup> The present results on the effects of DR agonists on cAMP levels may suggest that both D<sub>1</sub>-like and D<sub>2</sub>-like DR are functionally active in human lymphocytes; however, that D<sub>1</sub>-like DR

stimulation may result in stronger effects than D<sub>2</sub>-like DR stimulation at the second messenger level. Moreover, the correlation between D<sub>1</sub>-like DR-induced cAMP increase and DR score suggests that the DR genetic profile may affect the ability of lymphocytes to respond to dopaminergic agents.

**Table 2.** Dopaminergic receptor (DR) gene variants and regulatory T (Treg) inhibition of effector T (Teff) cell proliferation

Gene	Variant	Genotype	No. of participants	Treg-induced inhibition (%)	ANOVA	P for trend
<i>DRD1</i>	rs4532	A/A	2	-57.7 ± 3.3	0.005	0.004
		A/G	8	-54.3 ± 5.0		
		G/G	2	-36.5 ± 8.8		
	rs686	C/C	4	-58.4 ± 5.4	0.066 <sup>1</sup>	na
		C/T	7	-49.5 ± 8.9		
T/T		1	-42.8			
<i>DRD2</i>	rs1800497	G/G	8	-52.4 ± 10.4	0.824 <sup>1</sup>	na
		G/A	4	-51.1 ± 5.6		
		A/A	0	=		
	rs6277	C/C	3	-58.9 ± 7.02	0.115 <sup>1</sup>	na
		C/T	8	-49.2 ± 8.9		
		T/T	1	-52.5		
<i>DRD3</i>	rs6280	G/G	2	-41.4 ± 15	0.152	0.059
		G/A	5	-52.4 ± 5.5		
		A/A	5	-55.7 ± 6.1		
<i>DRD4</i>	7 48-base pair VNTR	7R/7R	9	-53.1 ± 6.1	0.458 <sup>1</sup>	na
		7R/4R	3	-48.5 ± 15.9		
		4R/4R	0	=		

ANOVA, analysis of variance; na, not applicable.

<sup>1</sup>By two-tailed Student's *t*-test.

The clear correlation between the DR score and Treg-induced reduction of Teff cell proliferation strengthens previous observations about the inhibitory role of D<sub>1</sub>-like DR on human Treg cells.<sup>3</sup> A higher DR score indicates increased D<sub>1</sub>-like DR activity (as confirmed by cAMP increase in total lymphocytes), and probably results in reduced regulatory functions by Treg cells. Indeed, Treg cells from individuals with higher DR score exert less inhibition on Teff cell proliferation (Fig. 1d). The DR gene variant that shows the strongest association with Treg cell function is *DRD1* rs4532. In our previous study,<sup>6</sup> we described a correlation between *DRD1* rs4532 and DR D<sub>1</sub> expression on T lymphocytes. In particular, individuals carrying the *DRD1* rs4532 G/G genotype had  $0.21 \pm 0.05 \times 10^9$  DR D<sub>1</sub> + CD4<sup>+</sup> T cells/ml, individuals with the G/A genotype had  $0.11 \pm 0.04 \times 10^9$  DR D<sub>1</sub> + CD4<sup>+</sup> T cells/ml, and the only one individual with the A/A genotype had  $0.03 \times 10^9$  DR D<sub>1</sub> + CD4<sup>+</sup> T cells/ml. In the present study, the participants with the G/G genotype had the lowest Treg cell function whereas the A/A genotype individuals showed the highest Treg cell function (Table 2). We previously showed that dopaminergic inhibition of Treg cell function occurs through the activation of D<sub>1</sub>-like DR,<sup>3</sup> and in agreement with those findings we now observed that the lowest Treg cell function is associated with the highest expression of D<sub>1</sub>-like DR D<sub>1</sub>, whereas the highest Treg cell function occurs when almost no D<sub>1</sub>-like DR D<sub>1</sub> are expressed on CD4<sup>+</sup> T cells. Preliminary experiments with DA confirmed the ability of this catecholamine to reduce Treg-induced

inhibition of Teff cell proliferation,<sup>3,8</sup> and also possibly suggested that DA-dependent reduction of Treg cell function is dependent on the availability of D<sub>1</sub>-like DR D<sub>1</sub>, because it was higher in the A/G and G/G genotypes and lower in the A/A genotype.

Results raise the issue of possible relationships between *DRD1* genetic variants and Treg cell dysfunction-related disease. Interestingly, *DRD1* genetic variants have been associated with cancer, but it is unclear whether Treg cells contribute.<sup>12</sup> More investigations are needed in cancer as well as in autoimmunity, organ transplantation and infectious disease, because increasing the knowledge about the role of DR genetic variants in the modulation of T lymphocyte function and in particular of Treg cells might result in better understanding of individual variability of immune responses in health and disease, providing the basis for the development of tailored therapeutic approaches.

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### Author contribution

MC and FM contributed to the study conception and design; MF, NK and ER contributed to the acquisition of data; and MC, NK, MF and FM performed the analysis and interpretation of data. 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 that they have confidence in the integrity of the contributions of their co-authors.

### Disclosure

The authors declare that they have no relevant conflicts of interest.

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